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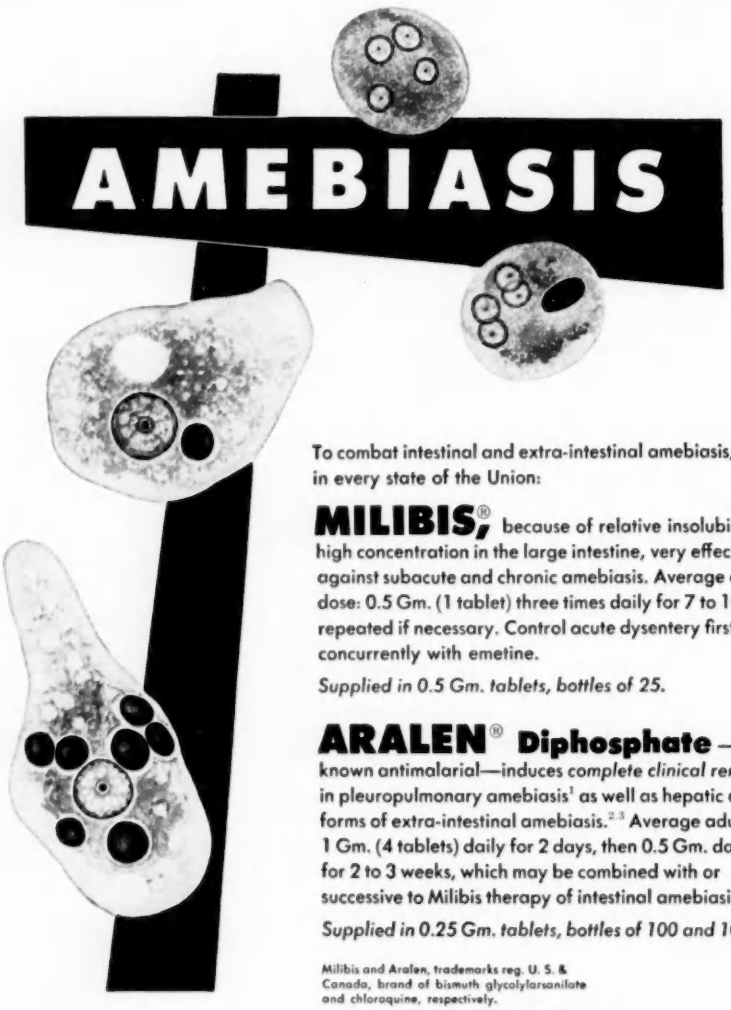

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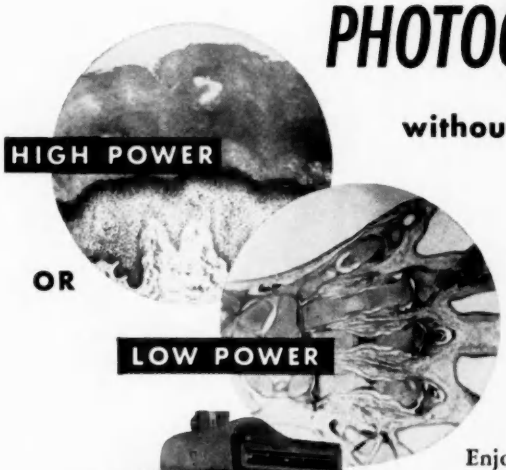

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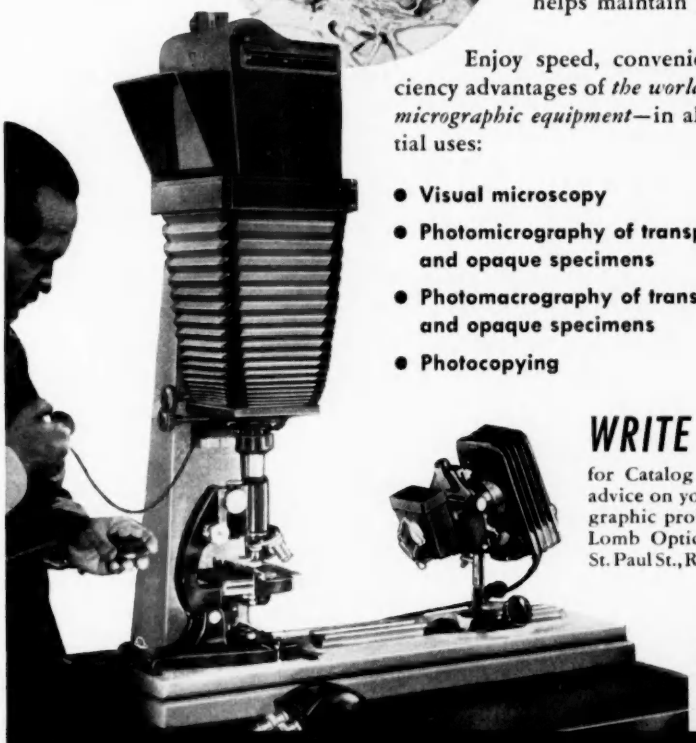

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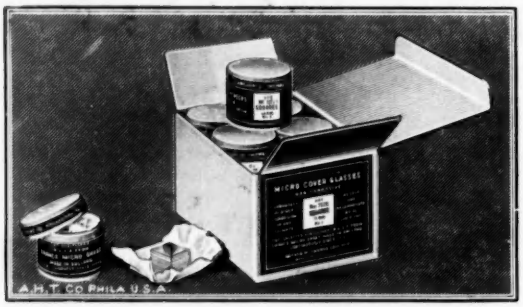

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DON E. EYLES, FRANCES E. JONES AND CLINTON S. SMITH

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The work of Meleney and his associates (1930, 1932) has given a general picture of the prevalence of *Endamoeba histolytica* and other intestinal parasites in the rural sections of Tennessee. In addition these investigators provided detailed data on one small community (Milam and Meleney, 1931) but aside from these studies no data are available with regard to the epidemiological pattern of occurrence of these parasites in the State of Tennessee.

This report concerns the epidemiology of *E. histolytica* and other intestinal parasites in a rural community located in the southern part of Fayette County in West Tennessee. Our plan was to determine prevalence rates and correlate these with observations made on the environmental and economic conditions in the community, studying the household as a unit. The findings were then to be studied to determine how they fit in with the currently accepted theories as to the transmission of the parasites.

DESCRIPTION OF THE AREA

The community studied is located in the southern part of Fayette County south of the Wolf River and north of the Mississippi State line. Its extent and location are shown on the map (Figure 1). The area of approximately 25 square miles supports a few more than the 2657 persons included in our study, 90 per cent of whom are of the Negro race.

The countryside is gently rolling and mostly open. The soil is characteristically a clayey loam much of which has become eroded due, at least in part, to improper agricultural practices. Absentee ownership is frequent and the economy of the region revolves largely around cotton farming under the tenant and crop sharing systems. The economic level of the majority of the people is extremely low and the maintenance of most of the houses, especially those of the Negro population, has been badly neglected. Illiteracy is frequent; the Negro schools are extremely poor in facilities and are not well attended.

EPIDEMIOLOGICAL METHODS

Each household in the area selected for study was paid a preliminary visit to gain the cooperation of the family in furnishing fecal specimens for examination, and to make observations as to the economic and environmental conditions and personal habits of the family members. At this time a group of families composed of the approximate number of individuals who could be examined concurrently

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by the laboratory were instructed as to the manner of supplying the specimens. Observations were continued in conjunction with the collection of these specimens and each household was observed by two or more staff members during the course of the study.

This was continued from September, 1950 to September, 1951, during which time the selected area was covered. More than 95 per cent of the families in the area cooperated and we succeeded in securing three specimens from almost all individuals.

At the time of the various visits to the household, the following data were recorded on a mimeographed sheet and later transferred to a marginal punch type coded card: 1. A list of all the family members with race, sex, age, education, and occupation. 2. Observations on fecal pollution of premises. 3. Data on fecal

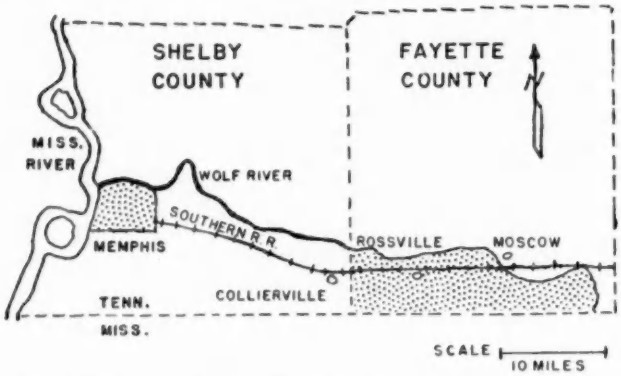

FIG. 1. Map of Shelby and Fayette Counties. Shaded area in Fayette County shows location and extent of the community included in this study.

disposal facilities. 4. Size and condition of house, with special attention to fly-proofing. 5. Observations on personal cleanliness of individuals in the household. 6. Observations on the degree of cleanliness of the house, especially the kitchen. 7. The type of water source and the result of test for pollution.

A number of other items were noted including the nature of the major crop (in the case of those farming), facilities for bathing and laundry, use of insecticides, etc., but the community was so uniform with respect to many of these factors that epidemiological analysis was not possible.

Some attempt was made to record symptoms of illness among the family members in order to obtain data for correlation with parasitological findings. These data were worthless, due probably to the low educational level of the community.

PARASITOLOGICAL METHODS

The parasitological findings in the study were based for the most part on the examination of three fecal specimens from each individual. A very few individuals furnished only two specimens but were included in the analyses. These specimens

were collected at weekly intervals in cylindric cardboard cartons of the type usually used for packaging ice cream. They were brought into the laboratory in portable ice boxes on the day of collection and refrigerated until examination the next day. Collections were usually not made on Friday to avoid the necessity of weekend refrigeration.

Due to the delay in examination made necessary by the method of collection of the specimens, no effort was made to find trophozoites and only examinations for cysts were made. A direct smear of the feces in iodine was examined, and a modification of Faust's zinc sulfate concentration technique was carried out to provide material for a second examination.

The concentration was effected by suspending a portion of the feces (about 0.5 gram) in approximately eight ml. of tap water contained in a shell vial. After stoppering, the material was thoroughly mixed by shaking, then strained through two thicknesses of damp gauze into a 12 by 100 mm. tube. This suspension was centrifuged at 1800 r. p. m. for 45 seconds. The supernate was then decanted and zinc sulfate solution (specific gravity, 1.180) was added to near the top of the tube. After resuspension of the fecal material by agitation a sufficient amount of zinc sulfate solution was added to produce a convex meniscus. The tube was allowed to stand for 10 minutes, after which a slide was touched to the top of the meniscus, then carefully inverted. The material thus obtained was stained with iodine and examined.

In the case of both preparations all of the material under the cover slips was examined. Experienced personnel made the examinations and *E. histolytica* cysts were verified under oil immersion, as were any other cysts which could not be positively identified with lower magnifications. A sufficient number of *E. histolytica* cysts in positive specimens were measured with a filar micrometer eyepiece to determine the relative incidence of the large and small races. Cysts measuring 10 micra or more were considered large race; those less, small race.

FINDINGS

Observed prevalence rates. Stool specimens from a total of 2,657 persons constituting 532 households were examined for intestinal protozoan cysts and helminth eggs. The prevalence rates of intestinal protozoa for both individuals and households by race are given in Table 1.

Of the intestinal amebae, *Endolimax nana* was found most frequently, being present in nearly 30 per cent of the population. The next most frequent were *Endamoeba coli* with a prevalence rate of about 22 per cent and *E. histolytica* with 10.5 per cent. *Iodamoeba bütschlii* cysts were reported from only 3.9 per cent of the individuals. *Giardia lamblia* was the more frequently found flagellate, being present in about nine per cent of the population as compared with less than one per cent for *Chilomastix mesnili*. The type of examination used did not disclose the other flagellates.

As might be expected familial prevalence rates were much higher. Two-thirds of the families had one or more individuals with *E. nana*, half the families had *E. coli*, and nearly one-third had *E. histolytica*.

Fayette County lies in the portion of Tennessee in which Meleney *et al.* (1932) found only 6.8 per cent of the population infected with *E. histolytica*. Meleney's group based their prevalence report on a single fecal specimen from each individual. Technical methods also differed; consequently, we do not believe our estimate to be discordant. Comparison with Meleney's study must be made cautiously as it was done 20 years ago and embraces a large and probably heterogeneous area.

TABLE 1

Observed prevalence of intestinal protozoa in a rural population of Fayette County, Tennessee, by individuals and by families

	PREVALENCE BY INDIVIDUALS			PREVALENCE BY FAMILIES		
	Number examined	Number infected	Per cent infected	Number examined	Number infected*	Per cent infected
Endamoeba histolytica						
Negro population	2,392	249	10.4	470	144	30.6
White population	265	29	10.9	62	19	30.6
Both populations	2,657	278	10.5	532	163	30.6
Endamoeba coli						
Negro population	2,392	525	21.9	470	257	54.7
White population	265	65	24.5	62	32	51.6
Both populations	2,657	590	22.2	532	289	54.3
Endolimax nana						
Negro population	2,392	705	29.5	470	318	67.7
White population	265	68	25.7	62	34	54.8
Both populations	2,657	773	29.1	532	352	66.2
Iodamoeba bütschlii						
Negro population	2,392	88	3.7	470	51	10.8
White population	265	15	5.7	62	9	14.5
Both populations	2,657	103	3.9	532	60	11.3
Giardia lamblia						
Negro population	2,392	214	8.9	470	128	27.2
White population	265	25	9.4	62	17	27.4
Both populations	2,657	239	9.0	532	145	27.2
Chilomastix mesnili						
Negro population	2,392	15	0.6	470	11	2.3
White population	265	1	0.4	62	1	1.6
Both populations	2,657	16	0.6	532	12	2.3

* A family was considered infected if one or more members carried the parasite in question.

The same conclusion applies in large measure to comparisons of the prevalences of the other species; however, the figure for *E. nana* given by Meleney *et al.* is markedly lower than that found by us, being 11.2 per cent as compared with our 29.1 per cent. We believe this discrepancy to be due to technical differences rather than a change in the prevalence of this species.

There appeared to be no significant differences in parasite prevalence related to race. This was surprising in view of the higher standard of living of the white race in the community.

Table 2 presents separately the frequency of the so-called small and large races of *E. histolytica*. The small race was more frequently observed in this community. This unusual finding is believed to be matter of fact and not a result of technique of examination inasmuch as another community (New Hope in middle Tennessee) studied by the same laboratory workers showed the usual much higher prevalence of the large race (Jones, Eyles, and Smith, 1953).

Relationship of observed prevalence to real prevalence. It has been universally recognized that the observed prevalence rates in a study of the type we have conducted do not reflect the real prevalence in a community. The degree to which the observed prevalence is lower than the actual prevalence is related to the efficiency of the technique of detection. We have attempted to use several published statistical methods of measuring our technique efficiency with varying and inconsistent results.

In a corollary study to the one being reported, we made repeated weekly examinations on a group of nearly 100 persons. From the parasitological data thus

TABLE 2

Observed prevalence of large and small races of E. histolytica *in Fayette County*

	LARGE RACE		SMALL RACE		BOTH RACES	
	Number*	Per cent of pop.	Number*	Per cent of pop.	Number	Per cent of pop.
Negro population	126	5.3	143	6.0	20	0.8
White population	15	5.7	17	6.4	3	1.1
Both populations	141	5.3	160	6.0	23	0.9

* This includes individuals having both races.

obtained we were able to graph the increase in the number of infected persons detected as the number of examinations became larger. After from 10 to 20 examinations few additional positives were observed and most of those could be considered new infections, although this is subject to some question.

Considering that 10 examinations demonstrated all or almost all of the infections, the efficiency of our three examinations per person was calculated empirically as follows: *E. histolytica*, 67 per cent; *E. coli*, 83 per cent; *E. nana*, 77 per cent; *I. bütschlii*, less than 67 per cent; and *G. lamblia*, less than 67 per cent. Using these efficiency estimates, the real prevalences of the three important ameba species were approximated as follows:

E. histolytica.....15.7 per cent
E. coli.....26.7 per cent
E. nana.....37.8 per cent

We believe that these figures represent a good estimate of the actual amount of parasitism in the community, but in the pages which follow we have used the observed prevalence in the statistical analyses.

Sex and age prevalence. The series of graphs shown in Figure 2 present a picture of the sex and age prevalence of the intestinal protozoa. It can be seen that as a

whole the female groups had the higher intestinal amebic rates. This higher rate of parasitism occurred in the adult age groups, whereas the prevalence was about the same for the two sexes under age 20. The difference between female and male

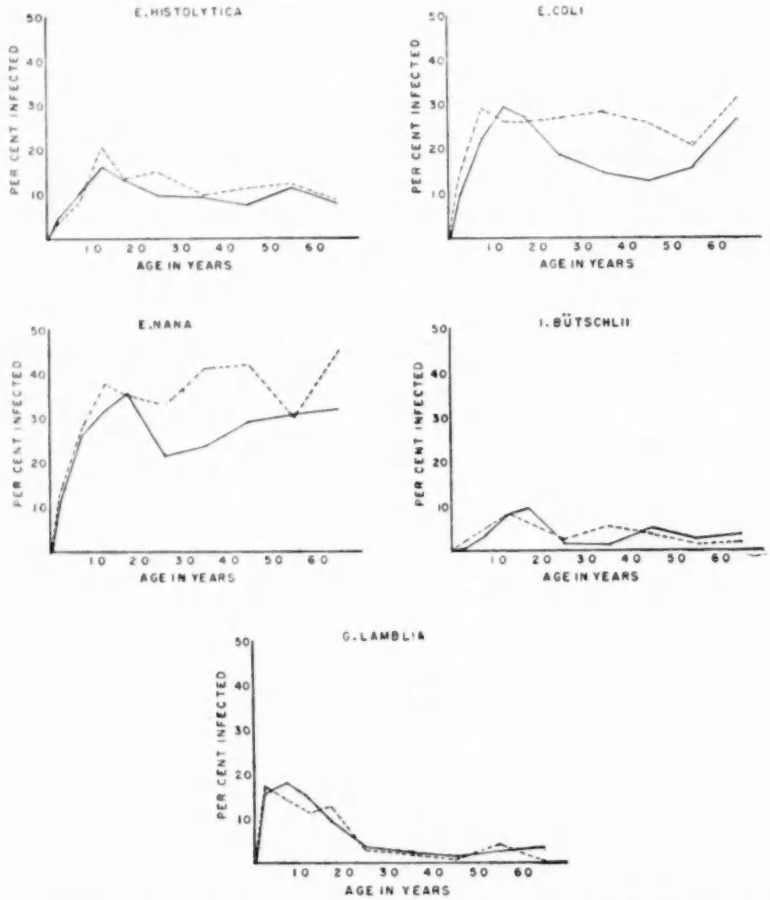

FIG. 2. Observed age prevalence of the intestinal protozoa. In the case of each parasite, the solid line represents the male sex and the dotted line the female sex.

prevalence was most marked for *E. nana* and was scarcely, if at all, noticeable for *I. bütschlii*. The difference was only slight for *E. histolytica*.

The intestinal amebae showed age distribution pattern similarities also. All but *E. nana* were most frequent in the 10 to 14 year age group. *E. nana* showed its absolute highest incidence in the age group over 60 years but was uniformly high for all age groups over 10 years. The amebae other than *E. nana* showed a

marked decline in prevalence after the maximum was reached in the 10 to 14 year age group; this was particularly noticeable in the male group.

Giardia was most prevalent in the 5 to 9 year age group and showed about the same prevalence in males and females in all age groups. Since few individuals had *Chilomastix*, no reliable age or sex distributions could be charted.

Due to the consistent finding of higher female prevalence in the adult groups, it is our opinion that we were dealing with a real difference. Our findings can be explained rationally on the following basis. In the first place, the similar prevalence rates in the children are probably due to the fact that there is little difference between the sexes as as regards sanitary habits. Both groups tend to be untidy and it is only as maturity is approached that their habits become different. As adulthood is reached the female groups become involved with household duties which must expose them to greater risk of infection, whether transmission be by direct contact or by an indirect method. The females tend to remain in the home. They handle children, wash clothes, and perform other tasks which might involve the risk of contamination.

Some investigators have not found sex differences. Probably clear sex differences do not occur in all communities. Our finding is similar but more marked than that of Svensson (1935) in a rural Swedish group. On the other hand, Svensson concluded that in an urban group, the males tended to have a higher rate of parasitism.

The implications with regard to the mode of transmission are not entirely clear, but the finding of greater prevalence in the female population would seem to be in best accord with transmission by direct contact with infected persons or by contact with contaminated articles. It would seem that transmission by the contamination of food by handlers or flies would result in similar sex prevalences, as would transmission by the contamination of the water supply. The difficulty is that we cannot be assured that a single principal mode of transmission exists.

The age prevalence pattern which we found is dissimilar to that reported by Meleney *et al.* (1932) from the State of Tennessee as a whole and also differs from the pattern in New Hope community (Milam and Meleney, 1931). In the case of these two studies the prevalence rates were increasingly high in adulthood, falling only when old age was reached. In our study it was pointed out that prevalence for all species was greatest in preadulthood and fell more or less markedly in the various species during early adulthood. A similar pattern was described by Andrews (1934) in a Mexican community of low sanitary level.

We believe that this age prevalence pattern fits in with the hypothesis of transmission by direct contact or contact with contaminated articles or matter. Certainly the children are less conscious of the importance of personal cleanliness and good sanitation and would as a result be more likely to expose themselves to contamination. If transmission were through the medium of the handling of food, it would seem that such age differences would not exist; the same reasoning would apply to fly or water transmission.

The age prevalence changes would indicate that either a large number of spontaneous cures of amebiasis are occurring or that demonstrability is lower

in adulthood, possibly due to a developed tolerance to the parasites. We have evidence not yet published which indicates that demonstrability is not lower in adulthood and that the lower adult rates must be due to spontaneous cure. We can see no other logical explanation, despite the assertion by some (for instance d'Antoni, 1952) that spontaneous cure of *E. histolytica* infection does not occur. It should be added that in this community amebiasis is rarely diagnosed and treated and the efforts of physicians could not have contributed to our finding.

Distribution of initial cases. In an effort to determine the mode of entry of *E. histolytica* and the other amebic parasites into the individual households, families were studied in which only one member was parasitized by the species in question. Tabulations were made for the three important amebae and, by way of contrast, *Giardia*.

The original or initial amebic cases occurred more frequently in the females as is shown in Table 3. In contrast, *Giardia* had practically the same prevalence in both sexes. For each species the sex distribution of the initial cases was very similar to the sex distribution of all cases. In other words, just as the females as a whole had more cases of amebic parasitism, so did the females appear to contract the initial case in the family most frequently.

Table 3 also presents the age distribution of these initial cases. It is seen that initial cases occurred most frequently in adults, thus differing from the overall prevalence which was greatest in teen-age children. To a great extent this must be due to the fact that the single cases tended to occur more frequently in the smaller families which had fewer children, than in the larger families where the chances were greater that more than one case would occur. One can conclude, however, that persons of all ages may be instrumental in introducing the parasites into the household. The large number of initial cases in the adults of 50 and over may be significant, although we cannot at present adequately interpret this finding.

We believe that the initial case findings fit in well with the hypothesis of contact transmission. The females undoubtedly have the closest contact with neighboring families and with relatives. Furthermore, if food handling were the principal mode of transmission we should expect as many initial cases, if not more, in the males since the males probably eat away from home more frequently than the females. Likewise, it would seem that fly or water transmission would similarly affect both sexes.

Familial prevalence. The impression was gained from the parasitological work that some families had more than the expected number of persons with amebic parasites. We had, for instance, one family of 15 with 10 *E. histolytica* infections and more than 10 infections with *E. coli* and *E. nana*. There were a number of other families with four to six infections. That such familial prevalence occurs has been asserted by many workers, but a few believe the finding of a number of infected individuals in one family is due to chance.

In order to test this statistically, the expected number of infections by each parasite for each family was calculated from the overall prevalence rates, then a summation of chi-square ($[O - E]^2/E$) was made and the probability that such

TABLE 3

Comparison of sex and age distribution of initial cases with distribution of all cases

	NUMBER OF INDIVIDUALS*	*Endamoeba histolytica*				*Endamoeba coli*				*Endolimax nana*				*Giardia lamblia*			
		Initial cases		All cases		Initial cases		All cases		Initial cases		All cases		Initial cases		All cases	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Males	1316	45	3.4	129	9.8	57	4.3	256	19.4	69	5.2	338	25.7	48	3.6	124	9.4
Females	1341	60	4.5	149	11.1	93	6.9	334	24.9	84	6.3	435	32.4	44	3.3	115	8.6
Total	2657	105	3.9	278	10.5	150	5.6	590	22.2	153	5.8	773	29.1	92	3.5	239	9.0
Age groups																	
0–4	473	6	1.3	20	4.2	6	1.3	57	12.0	8	1.7	58	12.3	24	5.1	78	16.5
5–9	372	8	2.1	35	9.4	18	4.8	96	25.8	6	1.6	102	27.4	18	4.8	60	16.1
10–14	334	14	4.2	61	18.3	18	5.4	93	27.8	5	1.5	116	34.7	14	4.2	43	12.9
15–19	271	12	4.4	36	13.3	19	7.1	72	26.6	20	7.4	96	35.4	19	7.0	30	11.1
20–29	272	15	5.5	35	12.9	22	8.1	64	23.5	21	7.7	77	28.3	5	1.8	9	3.3
30–39	282	14	5.0	27	9.6	14	5.0	62	22.0	28	9.9	94	33.3	4	1.4	6	2.1
40–49	249	12	4.8	24	9.6	20	8.0	49	19.7	24	9.6	89	35.7	1	0.4	3	1.2
50–59	186	12	6.4	22	11.8	8	4.3	33	17.7	19	10.2	57	30.6	5	2.7	6	3.2
60+	214	12	5.6	18	8.4	25	11.7	62	29.0	22	10.3	82	38.3	2	0.9	4	1.9

* The ages of four individuals were not ascertained and these are included only in the tabulation by sex.

findings were due to chance was determined from chi-square tables. The calculations resulted in large chi-square values indicating the findings could not have been due to chance. Thus cases of parasitism do tend to occur in particular families. This may be due in part to the presence in these families of more persons in the age group showing the highest amebic prevalence. However, the large chi-square values probably could not be accounted for by this occurrence. More likely, environmental conditions within the household which allowed some infections to occur made additional infections more probable.

TABLE 4

Prevalence of intestinal helminths in a rural population of Fayette County, Tennessee, by individuals and by families

	PREVALENCE BY INDIVIDUALS			PREVALENCE BY FAMILIES		
	Number examined	Number infected	Per cent infected	Number examined	Number infected	Per cent infected
Ascaris lumbricoides						
Negro population	2,392	109	4.6	470	58	12.3
White population	265	4	1.5	62	4	6.5
Both populations	2,657	113	4.3	532	62	11.6
Necator americanus						
Negro population	2,392	3	0.1	470	3	0.6
White population	265	1	0.4	62	1	1.6
Both populations	2,657	4	0.2	532	4	0.8
Trichuris trichiura						
Negro population	2,392	1	0.04	470	1	0.2
White population	265	0	0.00	62	0	0.0
Both populations	2,657	1	0.04	532	1	0.2
Hymenolepis nana						
Negro population	2,392	15	0.6	470	11	2.3
White population	265	6	2.3	62	3	4.8
Both populations	2,657	21	0.8	532	14	2.6
Hymenolepis diminuta						
Negro population	2,392	3	0.1	470	1	0.2
White population	265	0	0.0	62	0	0.0
Both populations	2,657	3	0.1	532	1	0.2

This finding fits in satisfactorily with the theory that transmission is mainly by direct or indirect contact. The number of potential transmitting contacts would become greatly increased after the initial infection entered the family, as contacts within the family would be more frequent and more intimate than those outside the household.

Prevalence of helminth parasites. The laboratory findings showed the prevalence rates of the helminth parasites to be very low in the community covered by our study. This conforms with a previous report (Otto, Cort and Keller, 1931) that these parasites occurred much less frequently in the western portion of the state than in the other sections. Table 4 gives these prevalence rates for individuals and families by race. As in the case of the protozoa, the family prevalence rates of the helminth parasites were higher than the individual rates.

Ascaris lumbricoides, the helminth most frequently found, occurred in 4.3 per cent of the individuals with a prevalence rate in the Negro population three times as high as in the white. *Necator americanus* was rarely found, occurring in only 0.2% of the individuals, while only one person was found to harbor *Trichuris trichiura*. *Hymenolepis nana* was found to be second to *Ascaris* in frequency of occurrence with a prevalence rate of 0.8%. In contrast to *Ascaris*, this parasite infected whites four times as frequently as Negroes; however, chance may have given rise to this great difference as the numbers involved were small.

One Negro family was found to have three members harboring the rat tapeworm, *Hymenolepis diminuta*, which is rarely found in human beings. A visit was made to this home but no insects were observed which could explain the infections in this family alone. Grain beetles in particular were sought but were not found.

TABLE 5

Frequency of multiple infection with intestinal protozoa as compared with expected frequency (assuming random distribution)

SPECIES	OBSERVED NUMBER OF MULTIPLE INFECTIONS	EXPECTED NUMBER OF MULTIPLE INFECTIONS	RATIO OF OBSERVED AND EXPECTED NUMBERS	PROBABILITY THAT ASSOCIATION WAS DUE TO CHANCE
E. histolytica and E. coli	139	62	2.24	<.0000006
E. histolytica and E. nana	175	81	2.2	<.0000006
E. histolytica and I. bütschlii	38	11	3.4	<.0000006
E. histolytica, E. coli, and E. nana	101	18	5.6	<.0000006
E. histolytica, E. coli, E. nana, and I. bütschlii	23	1	23.0	<.0000006
E. histolytica and G. lamblia	26	25	1.0	.75*
E. histolytica and Ascaris	9	11	0.8	.63*

* Approximated by interpolation in chi-square table.

Only *Ascaris* was found frequently enough to permit correlation with observations on the economic and environmental conditions of the households. The data are included in Table 6 along with data on the protozoa and are discussed along with the protozoa.

Prevalence of mixed infections and association of parasites. Mixed infections of the intestinal amebae and mixed infections of the amebae with the flagellates and helminths were frequent. Table 5 shows the prevalence of these mixed infections and compares the observed prevalence with that expected, assuming strict independence of occurrence (See Boughton and Byrd, 1938, for method). It is clear that the multiple infections of the amebae occur much more frequently than chance occurrence would permit. This is especially true when the group harboring all four intestinal amebae is considered. In this instance there were 23 multiple infections observed, whereas only one was expected. Similarly, multiple infections of the three common amebae, *E. nana*, *E. coli* and *E. histolytica*, were found in 101 individuals compared with an expected 18. Infection with two amebic species occurred in more than twice the expected number of cases; for instance, 139 infections with *E. coli* and *E. histolytica* were observed compared with 62 expected.

The probabilities of such differences occurring by chance are infinitesimal. On the other hand the observed number of infections of the amebae with *Giardia* and with *Ascaris* did not materially, if at all, exceed the expected number.

The data presented above are in remarkable accord with those of Boughton and Byrd (1938). In their study also, the observed number of double infections was a little more than twice the expected number. It would appear that this pattern of occurrence is characteristic of a variety of populations as we have observed it in another locality (Jones, Eyles and Smith, 1953) and Boughton and Byrd used not only their own Georgia data but also data from Boeck and Stiles' (1923) study, which came from a variety of sources.

Boughton and Byrd (1938) discussed the possible significance of this phenomenon. Two hypotheses which would serve to explain the findings are: 1. That certain individuals are more susceptible to the amebae than others; and 2. That certain individuals are more exposed to infection by virtue of unsanitary habits or other causes. It is easy to assume that the second hypothesis provides the better explanation; however, if exposure to risk of infection be the answer, it is difficult to understand why a greater degree of association between the amebae and *Giardia* was not found if the same mode of transmission is assumed. In our group of people, the difference in age incidence for the amebae as compared with *Giardia* might provide some explanation for the lack of association, but Boughton and Byrd, who reported a similar low degree of association, were dealing largely with adults in whom the age differences are not notable. Certainly, the occasional presence in heavily parasitized families of individuals with none of the intestinal amebae might possibly be due to less susceptibility to infection on the part of these individuals. Variation in susceptibility may be common to the four species of amebae.

On the other hand, the occurrence of persons with multiple infections is in good accord with the theory of transmission by direct contact or contact with contaminated matter. The unsanitary individual would have the greater number of potential infective contacts, and would be more likely to become infected with more than one amebic species. Experimental work on individual susceptibility is needed for better interpretation of the findings.

Correlation of parasitological findings and environmental conditions. In order to obtain leads as to the possible modes of transmission of intestinal parasites in the study area, observations were made of environmental conditions in and around the houses. These have been treated statistically by several methods and are summarized in Table 6. In some instances the classifications were arbitrary and borderline cases could have been placed in different groups with equal justice but the difference between the extreme classifications was very great.

Fecal pollution of premises was looked for during the course of four or more visits to the families. In the case of each parasite species, the families with clean premises had the lowest parasite prevalence rates. In the case of all except *E. histolytica*, the families with the heaviest pollution also had the highest parasite prevalence. The differences in prevalence between the group with clean premises and the group with polluted premises was statistically significant col-

TABLE 6

Correlation of parasitological findings and environmental conditions

	NUMBER OF FAMILIES IN EACH CATEGORY*	*Endamoeba histolytica*		*Endamoeba coli*		*Endolimax nana*		*Iodamoeba bütschlii*		*Giardia lamblia*		*Ascaris lumbricoides*	
		Number infected	Per cent infected	Number infected	Per cent infected	Number infected	Per cent infected	Number infected	Per cent infected	Number infected	Per cent infected	Number infected	Per cent Infected
Fecal pollution of premises													
None	157	29	18.5	71	45.2	96	61.1	14	8.9	27	17.2	7	4.5
Moderate	230	86	37.4	126	54.8	153	66.5	26	11.3	61	26.5	27	11.7
Heavy	144	47	32.6	91	63.2	102	70.8	20	13.9	57	39.6	28	19.4
Fecal disposal													
Indoor plumbing or sanitary privy	113	21	18.6	60	53.1	64	56.6	8	7.1	29	25.7	10	8.8
Unsanitary privy	300	107	35.7	167	55.7	208	69.3	37	12.3	85	28.3	34	11.3
No facilities	119	35	29.4	62	52.1	80	67.2	15	12.6	31	26.0	18	15.1
House cleanliness													
Clean	54	9	16.7	25	46.3	30	55.6	5	9.3	9	16.7	2	3.7
Fair	298	96	32.2	156	52.3	200	67.1	29	9.7	67	22.5	28	9.4
Dirty	180	58	32.2	108	60.0	122	67.8	26	14.4	69	38.3	32	17.8
Personal cleanliness													
Clean	64	12	18.8	28	43.8	34	53.1	8	12.5	10	15.6	2	3.1
Fair	301	98	32.6	160	53.2	204	67.8	27	9.0	69	22.9	29	9.6
Dirty	167	53	31.7	101	60.5	114	68.3	25	15.0	66	39.5	31	18.6
Water supply													
On premises	327	110	33.6	180	55.0	219	67.0	34	10.4	100	30.6	45	13.8
Off premises	205	53	25.8	109	53.2	133	64.9	26	12.7	45	22.0	17	8.3
Unpolluted	188	59	31.4	98	52.1	113	60.1	16	8.5	49	26.1	25	13.3
Polluted	340	102	30.0	189	55.6	235	69.1	43	12.6	95	27.9	37	10.9
Family size (Individuals)													
1–3	192	35	18.2	71	37.0	103	53.6	13	6.8	18	9.4	3	1.6
4–6	201	59	29.3	117	58.2	136	67.7	19	9.4	60	29.8	26	12.9
7–9	107	49	45.8	75	70.1	85	79.4	16	14.9	50	46.7	27	25.2
10+	32	20	62.5	26	81.3	28	87.5	12	37.5	17	53.1	6	18.8

* The total number of families in the various categories is not the same due to the fact that it was not always possible to obtain complete data on each family.

lectively for all of the parasites and individually for all species except *I. bütschlii* (tested by means of chi-square). The difference in prevalence between families with a moderate amount of pollution and the families with much pollution was not great for the amebic parasites, but was very marked for *Giardia* and *Ascaris*.

The findings with regard to pollution are in accord with the hypothesis of transmission by contact for the amebic species. The *Ascaris* findings fit in well with the long held belief that premises pollution is a major factor. The findings are not inconsistent with water transmission as the promiscuous deposit of fecal matter over the premises could easily lead to water contamination. Likewise, the pollution might provide an opportunity for flies to become infected.

The pollution findings do not accord quite so well with the "food handler" hypothesis. However, in considering these environmental correlations, reservations must be made as we are dealing with factors which may be linked; families with good privies are less likely to pollute the premises than those with poor privies or with no privies. Moreover, clean families are likely to have good facilities for fecal disposal, are not likely to pollute the premises, and are in all probability more enlightened in general.

The facilities for fecal disposal were observed for each household. Where good facilities, either indoor plumbing or sanitary privies, were present, the parasite rates were slightly to distinctly lower than where poor unsanitary privies or no privies were present. In the case of the amebae, families with no privies had slightly lower parasite prevalence rates than families with unsanitary privies; however, the differences were not statistically significant. In the case of *Ascaris* the families with no privies had a higher rate of parasitism, but again the difference was only suggestive and not statistically significant.

Families with good facilities for fecal disposal were in general clean, so it is not surprising that lower parasite prevalence was found. On the other hand the fact that families with no privies had no more or possibly fewer infections than families with poor privies may indicate that poor privies provide a source of contamination. In contrast, the *Ascaris* rate was highest in the no privy group, a finding that, although in itself not statistically significant, is in accord with many previous studies of this parasite.

During the various visits to the households, each was classified as to the cleanliness of the house and the cleanliness of the family members. Families maintaining standards similar to those of middle-class families in urban areas were classified clean. Those which maintained a standard of cleanliness below the aforesaid, but good when the poor facilities for implementing cleanliness were considered, were classed as fair. The poor category included the families which made little or no effort to keep their persons or surroundings clean. The personal and household cleanliness were very closely correlated, the dirty persons having dirty houses and *vice versa*. As a consequence, the parasite prevalence rates were similar in the two groupings.

In the case of all of the parasites, the clean families tended to have the lowest parasite prevalences, the fair families intermediate prevalence rates and the dirty families the highest parasite prevalence rates. *I. bütschlii* presented an exception

in that the clean families were infected at a slightly higher rate than the fair, but this was most likely a chance occurrence. In the case of the amebae taken collectively there was a statistically significant difference in prevalence rates between the clean and the fair families, but the difference between the fair and the dirty families was not statistically significant.

In the case of *Ascaris* and *Giardia* the differences between clean and fair on one hand and fair and poor on the other were statistically significant. *Ascaris* was actually more than five times as prevalent in the dirty families than in the clean, and about twice as prevalent in the dirty as the fair.

The fact that the clean families had lower prevalences of amebae is consistent with the theory of contact transmission, but the finding of only small, possibly unreal, differences between the fair and dirty families would not appear to be consistent. This anomalous finding would appear to be inconsistent with the fly and water theories also, as dirty families would be expected to have more flies and be expected to pollute the water more frequently than the clean families. Our observations were of general cleanliness and did not measure the degree of fastidiousness with regard to fecal contamination specifically. It is conceivable that general cleanliness is not closely correlated with care in protecting against fecal contact, but this does not seem likely.

Water supply was also studied and has been considered in another report (Johnson, 1953). Some additional statistical analyses based on this work are included in Table 6. It can be seen that ameba prevalence was slightly but not significantly greater in those families whose water supply was found to be polluted (presumptive *Escherichia coli* test). Whether or not these small differences have any real meaning is doubtful. Comparison between families that had water on the premises and those who had to carry water from a distance disclosed that the latter had slightly lower amebic prevalence rates. Chance could easily account for these differences, but in the case of *Giardia* the rate was distinctly lower.

One factor which was strongly correlated with amebic prevalence was size of family. As Table 6 shows, if family size exceeded six the amebic prevalence was found to be much higher for all species. This might be due either to the increased number of intimate contacts caused by the greater number of individuals or the increased intimacy of contact due to overcrowding. The finding is therefore in harmony with the hypothesis of contact transmission.

DISCUSSION

There is a wide diversity of opinion with respect to many of the concepts regarding amebiasis. This is due to a scarcity of evidence bearing on many points of disagreement and to the difficulty of interpreting much of the evidence which is available. Undoubtedly, many persons will disagree with some of the interpretations we have suggested based on the findings of our study. Our hope is that we have provided additional facts which will prove useful in understanding some of the problems of amebiasis, and that our comments will stimulate thinking on these problems.

We have studied an entire community from an epidemiological point of view.

We have seen how amebiasis is distributed under natural conditions in this community. We have considered not only *E. histolytica* but the other amebae, for we believe the essential mode of transmission to be similar. For contrast, we have studied some of the other human intestinal parasites in the belief that the differences in manner of occurrence may be enlightening.

Discussion of the various findings especially as they bear on the transmission of the amebae has been included in the test, as we believed that separating the discussion from the findings in this paper would cause a lack of unity and make understanding difficult. The remarks here are limited to considerations on the study as a whole.

This study provided no clear cut evidence as to the mode of transmission in the community. We do not know positively that there is any single principal mode of transmission; actually, more than one mode may be important. As we presented our findings we have considered their bearing on the four commonly proposed hypotheses of transmission. With minor exceptions, our findings are in harmony with transmission by direct contact or by contact with contaminated articles. Certain findings indicate that the major role is not played by flies or by water. Other findings are not in accord with the food handler hypothesis.

One fact that we believe evident is that amebiasis is favored by poor standards of cleanliness and sanitation. The providing of good sanitary facilities and the means for maintaining cleanliness, along with the education necessary to ensure the use of these facilities, should result in a reduction in the rate of parasitism.

Poor as present standards are, the people in the community we have studied are undoubtedly becoming more conscious of sanitation and cleanliness. During the past few decades there are indications that the major prevalence of the amebae has swung from the adult group to the children. It may well be that increased awareness of the importance of cleanliness is the cause of this change.

SUMMARY AND CONCLUSION

An epidemiological study with respect to *Endamoeba histolytica* and other intestinal parasites in a rural community of West Tennessee resulted in the following:

1. Observed prevalence of *E. histolytica* was 10.5 per cent; *E. coli*, 22.2 per cent; *Endolimax nana*, 29.1 per cent and *Iodamoeba bütschlii*, 3.9 per cent; *Giardia lamblia*, 9.0 per cent and *Chilomastix mesnili*, 0.6 per cent.

2. The small race *E. histolytica* was slightly more frequent than the large race.

3. Actual prevalence rates were estimated to be about half again as high as the observed rates.

4. Familial rates were: *E. histolytica*, 30.6 per cent; *E. coli*, 54.3 per cent; *E. nana*, 66.2 per cent; *I. bütschlii*, 11.3 per cent; *G. lamblia*, 27.2 per cent; and *C. mesnili*, 2.3 per cent.

5. There were no significant differences in prevalence by race, which was surprising as the standard of living of the white race was much higher than that of the Negro.

6. The female sex in general had higher intestinal amebic prevalences, particularly in the adult age group. No sex difference was seen for *Giardia*.

7. *E. histolytica*, *E. coli*, and *I. bütschlii* were most prevalent in the 10 to 14 year age group. *E. nana* showed its highest absolute prevalence in the age group over 60 years, but was uniformly high for all age groups over 10 years. The amebae other than *E. nana* showed a marked decline in prevalence following the maximum, particularly in the male group.

8. *Giardia* was most frequent in the 5 to 9 year age group.

9. Initial cases in families were most frequent in females but occurred in all age groups.

10. Many families had more infections of the different parasites than the number expected through the operation of chance alone.

11. *Ascaris lumbricoides* was the most frequent helminth, occurring in 4.3 per cent of the individuals and 11.6 per cent of the families. Next was *Hymenolepis nana* which was found in 0.8 per cent of the individuals and 2.6 per cent of the families. A few isolated cases of parasitism by *Necator americanus*, *Trichuris trichiura*, and *Hymenolepis diminuta* were found.

12. Mixed infections of the several amebae were much more frequent than would be expected by the operation of chance alone. Double infections of *E. histolytica* and *G. lamblia* and *E. histolytica* and *Ascaris* were no more frequent than expected through chance alone.

13. Prevalence of the intestinal amebae was correlated with degrees of fecal pollution of premises, cleanliness of house and person, and to some degree with the quality of fecal disposal facilities. Prevalence was highly correlated with size of family. Pollution of water supply was not associated with significantly increased amebic prevalence.

An effort was made to interpret the findings with respect to the various hypotheses of transmission of the intestinal amebae. It was concluded that on the whole the findings were in best accord with transmission by contact with infected persons or with articles contaminated by infected persons.

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COMPARATIVE ACTION OF SELECTED AMEBICIDAL AGENTS AND ANTIBIOTICS AGAINST SEVERAL SPECIES OF HUMAN INTESTINAL AMEBAE[1, 2]

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INTRODUCTION

The search for an ideal amebicide continues and is complicated by biological problems correlated with the ecology of the parasites residing in the human large intestine. In this complex environment, for example, there may coexist five distinct species of amebae as well as other protozoa, myriad bacterial types and a milieu of widely varying nutrient composition. At present we have little fundamental knowledge of the role of any of these components, including a convincing explanation of the apparent nonpathogenicity of *Endamoeba histolytica* in the typical human host.

The testing of potential amebicidal agents has depended heavily upon *in vivo* screening, despite the expense and time involved. There is no question about the value of a reliable *in vitro* screening technique, but considerable difference of opinion exists concerning those tests which have involved *E. histolytica*. Published studies contain conflicting data regarding the efficacy of given agents and this has tended to discredit *in vitro* testing generally. Analysis discloses, however, that the techniques themselves have differed widely as to composition of the test media, the ameba-bacteria combinations used in the test cultures and the criteria employed in fixing amebicidal endpoints. Some of the issues, together with bibliographic references, have been summarized in a recent review (Balamuth, 1952), but it seems pertinent to indicate here the nature of the variations encountered. Tests have been conducted in diphasic and in all-fluid media of widely differing composition and pH. The amebae have been accompanied either by mixed bacterial flora, or by various single bacterial types, or by *Trypanosoma cruzi*. Some tests have been conducted under aerobic conditions, while others have resorted to anaerobic incubation. Through all these types of testing procedures additional variations have been imposed by fluctuations of the age and size of amebic inocula, volumes of media involved, and the duration of tests. Under these circumstances it seems reasonable to reserve judgment on the validity and usefulness of *in vitro* data until some standardization is introduced. In this connection it is of interest to note the rather extreme position taken recently by Dobell (1947), probably the dean among students of intestinal amebae, that ". . . there is no good evidence that any chemical which is specifi-

[1] The main body of this paper was presented at the annual meeting of the American Society of Tropical Medicine in Savannah, November, 1950.

[2] This investigation was supported (in part) by a research grant from the National Institutes of Health, Public Health Service. The expert technical assistance of Morgan Brent is acknowledged.

cally toxic to this amoeba (i.e., *E. histolytica*) *in vitro* is without curative value *in vivo*."

For some time we have been studying *in vitro* the complex of human intestinal amebae. The primary aim has been to uncover physiological differences which might give some insight into differences in pathogenicity and even in incidence of the several species. Comparative reactions to chemical agents (e.g., amebicides and antibiotics) offered one approach to the general problem. When reference to current testing procedures had disclosed the variability mentioned above, it was decided to undertake a thorough study. The dual objective of this particular study has been to obtain reliable comparative data not yet available for the range of species, and at the same time possibly to elucidate some factors essential to a standard testing procedure.

MATERIALS AND METHODS

The plan called for testing agents of two general kinds: representatives of chemical groupings used routinely as amebicides, and some antibiotics of widely different antibacterial properties. In the first category were emetine hydrochloride, Vioform (soluble), and two trivalent arsenicals developed in Anderson's laboratory (Anderson *et al.*, 1947): carbarsone oxide and one of its dithio derivatives (p-[Bis(carboxymethylmercapto)arsino]-phenylurea) bearing the Chemotherapy Center number 914.[3] Antibiotics which were subjected to thorough tests included prodigiosin (prepared in this laboratory by Morgan Brent) and aureomycin hydrochloride.[3] Concentrated stock solutions were prepared either in distilled water (emetine, Vioform, and aureomycin), 75% ethanol (prodigiosin), 0.5% sodium bicarbonate (C.C. no. 914), or 50% propylene glycol (carbarsone oxide). Dilutions were made in the test medium and experimental tubes were set up in ranges of concentration increasing usually by a twofold factor.

The test organisms included the range of human intestinal amebae maintained in our laboratory: two strains each of *Endamoeba coli*, *Dientamoeba fragilis*, and *Endolimax nana*, in addition to two cultures of the *NRS* strain of *E. histolytica*, one containing a mixed flora and the other *Aerobacter aerogenes* alone. The non-*histolytica* species were originally isolated and established locally in stock cultures by a method to be described elsewhere; *A. aerogenes* was the dominant member of the mixed flora present in these cultures.

The tests extended 48 hours in 10-ml. volumes of liver-eggyolk infusion (Balamuth, 1946). The inocula consisted of the pooled, centrifuged and resuspended sediment from 48-hour flask cultures. Amebae were counted on Spencer bright-line hemocytometers, while bacterial counts employed decimal-dilution plating on Bacto-Stock Culture Agar. As will become evident in the tables below, relatively enormous numbers of amebae were inoculated in order to insure conservative endpoints; in addition, experiments were designed to test the influence of the amount of inoculum on endpoints. During the experiments

[3] It is a pleasure to acknowledge the cooperation of Dr. H. H. Anderson of the University of California in providing the compounds used in the present study, together with some helpful suggestions.

it became evident that the pooling technique influenced the "quality" of the inoculum. Control of such factors as agitation of amebae, centrifugation, oxygenation of the medium, adjustment of inocula with sterile versus used media, etc. limited the variability, but in practice these factors proved less critical than reduction of free rice starch in the pool. Controlled tests showed that in the presence of excess rice starch fewer amebae of poorer appearance were recovered. It is considered that the sharply angular particles may grind and mutilate the amebae during centrifuging and pipetting operations.

The number of bacteria present in inocula varied widely since the pools were adjusted only with reference to amebae, but during the 48-hour tests any bacterial inhibition became readily evident. Since experience had shown that aerobic incubation was preferable except in cases of bacterial inhibition, only aureomycin was tested under both aerobic and anaerobic conditions. Anaerobiosis was maintained in this last case by sealing cultures with a mixture of melted petrolatum and paraffin ("vaspar").

Several criteria were employed in fixing the amebicidal endpoints. Amebae were recorded as simply present or absent at the end of 48-hour runs, but sediment from all negative and doubtful tubes was subcultured to verify endpoints. Sediment was usually implanted into sterile culture medium; however, in cases of distinct bacterial inhibition the culture medium contained 12-hour-old flora of the particular strain being checked. In addition, protocols were kept of relative numbers and condition of amebae. Effects upon bacteria were noted in two ways. Bacterial populations of negative tubes were compared with controls. These data were correlated in turn with oxidation-reduction potential curves of cultures under treatment as in a previous study (Balamuth and Brent, 1950). The object of this dual approach was to discover whether any agent was acting primarily against the bacterial flora.

RESULTS

1. *Comparative amebicidal activity of some selected chemical agents.* The summary data are incorporated into Tables 1–4, grouping each agent separately with the several strains of amebae tested. In all, 249 series of experiments were conducted and were distributed equally among the various species; each series covered a range of concentrations for a given drug, and the number of series per drug was as follows: emetine, 50; Vioform, 30; carbarsone oxide, 44; C. C. no. 914, 41; prodigiosin, 40; and aureomycin, 44.

The format of the tables was standardized to show the condition of the inoculum in terms of amebic and bacterial populations, and to indicate at amebicidal endpoints the viable bacterial populations and final pH of the test medium. Since the tests were conducted in 10-ml. volumes of liver-eggyolk infusion, the concentration of organisms per ml. is one-tenth of that shown in the tables. The growth of each control bacterial flora was determined. These data showed consistently close agreement with populations at the amebicidal endpoints for emetine (Table 1), which is representative of those agents exhibiting little antibacterial activity in the range of concentrations studied. The large variation in

bacterial inocula for different strains of amebae was due to concentrating varying numbers of stock cultures to ensure comparable amebic inocula. These variations would be expected to have little influence upon maxima, since the stationary phase of bacterial growth was reached in less than 48 hours and all inocula were of the same age. Long experience with our medium has shown that normal bacterial growth at 48 hours ranges between 10^8 and 10^9 per ml.

The data for emetine (Table 1) disclose some interesting points. It is clear that *E. histolytica* exhibited a strikingly differential susceptibility to the drug. It is further evident that the monobacterial culture proved less resistant than the mixed culture, independently of bacterial viability. It may be observed that none of the bacterial floras was affected adversely.

TABLE 1

Minimal amebicidal concentrations of EMETINE HYDROCHLORIDE *for several species of human intestinal amebae*

STRAINS OF AMEBAE	TOTAL INOCULA		RESULTS		
	Bacteria ($\times 10^7$)	Amebae ($\times 10^3$)	Amebicidal Conc. (μgm./ml.)	Bact. Pop. ($\times 10^7$)	pH
Endamoeba histolytica:					
NRS + mixed flora	25	300	4	315	7.4
NRS + *A. aerogenes*	316	260	0.5–1.0	730	7.4
Endamoeba coli:					
So strain	585	210	100–200	750	7.2
Jo strain	1400	235	>200	1172	6.7
Dientamoeba fragilis:					
Bi strain	156	330	100	1300	7.4
R strain	138	330	100	1140	7.3
Endolimax nana:					
T strain	85	300+	100	1030	7.5
Th strain	190	275	100	410	7.4

Test conditions: Aerobic incubation at 37°C. for 48 hr. in 10-ml. amounts of liver-egg yolk medium. Several experiments per strain.

In the case of Vioform (Table 2), all species reacted in approximately the same way. The comparative data on bacterial viability indicate that the end-points approached the threshold of toxic action against the associated floras.

Reactions to the two trivalent arsenicals are grouped together in Table 3. Carbarsone oxide proved distinctly more effective and indeed exhibited the broadest spectrum of average amebicidal action of any drug in the series. On the other hand, the thioarsenite was consistently more active than Vioform, a well-recognized therapeutic agent. Neither compound was bactericidal at its minimal effective concentration.

The two antibiotics are placed together for comparison in Table 4, despite their chemical independence. Prodigiosin ranked with the most effective agents tested, and was not bactericidal. A previous publication from this laboratory (Balamuth and Brent, 1950) had recorded the reactions to prodigiosin of the

NRS and *UC* strains of *E. histolytica*, disclosing that a monobacterial culture was two to four times more susceptible than a polybacterial combination. The discrepancy between the *So* and *Jo* strains of *E. coli* was correlated with a visible

TABLE 2

Minimal amebicidal concentrations of VIOFORM (soluble) *for several species of human intestinal amebae*

STRAINS OF AMEBAE	TOTAL INOCULA		RESULTS		
	Bacteria ($\times 10^7$)	Amebae ($\times 10^3$)	Amebicidal Conc. (μgm./ml.)	Bact. Pop. ($\times 10^7$)	pH
Endamoeba histolytica:					
NRS + mixed flora	29	305	100	115	7.1
NRS + *A. aerogenes*	820	300+	100	140	7.5
Endamoeba coli:					
So strain	490	160	100	225	7.3
Jo strain	500	220	100–200	292	7.0
Dientamoeba fragilis:					
Bi strain	100	325	200	505	7.3
R strain	70	310	100	215	7.5
Endolimax nana:					
T strain	35	390	200	160	7.1
Th strain	45	380	200	230	7.4

TABLE 3

Minimal amebicidal concentrations of CARBARSONE OXIDE *and* P-[BIS(CARBOXYMETHYLMERCAPTO)ARSINO]-PHENYL UREA (C. C. NO. 914) *for several species of human intestinal amebae*

STRAINS OF AMEBAE	TOTAL INOCULA		RESULTS WITH CARB. OXIDE			TOTAL INOCULA		RESULTS WITH C. C. NO. 914		
	Bact. ($\times 10^7$)	Amebae ($\times 10^3$)	Amebicidal Conc. (μgm/ml.)	Bact. Pop ($\times 10^7$)	pH	Bact. ($\times 10^7$)	Amebae ($\times 10^4$)	Amebicidal Conc. (μgm/ml.)	Bact. Pop. ($\times 10^7$)	pH
Endamoeba histolytica:										
NRS + mixed flora	25	300+	4	290	7.2	35	305	40	265	7.1
NRS + *A. aerogenes*	270	300+	4	1000	7.2	425	245	20	510	7.5
Endamoeba coli:										
So strain	785	230	4–10	730	7.1	490	160	40	790	7.2
Jo strain	1200	210	4	1450	6.7	485	235	20–40	1080	6.7
Dientamoeba fragilis:										
Bi strain	113	326	4	800	7.2	160	326	20	980	7.4
R strain	88	310	4	1100	7.1	90	310	20	440	7.3
Endolimax nana:										
T strain	59	320	10–20	360	7.3	35	320	40	395	7.4
Th strain	190	275	10	870	7.1	45	300	10–20	860	7.5

absorption of the reddish prodigiosin by the bacterial sediment in the former cultures. Since prodigiosin is relatively insoluble in water, possibly it was being removed from solution through some activity of the particular flora. The same

feature was not observed with *Endolimax*, which suggests that this species may be less susceptible.

The case of aureomycin provided the greatest surprise in the entire study, in view of published reports claiming distinct and direct amebicidal activity of this antibiotic (Hewitt *et al.*, 1950; Spingarn and Edelman, 1951; McVay, 1952; etc.). From our data it is evident that aureomycin proved least amebicidal of the agents tested. Anaerobic incubation during tests did not cause a distinct shifting of endpoints as in the experiments of Bradin and Hansen (1950). The endpoints were doubled only in the cases of both strains of *Dientamoeba* and the *T* strain of *Endolimax*, which is not considered significant in our experimental design and

TABLE 4

Minimal amebicidal concentrations of PRODIGIOSIN *and* AUREOMYCIN HYDROCHLORIDE *for several species of human intestinal amebae*

STRAINS OF AMEBAE	TOTAL INOCULA		RESULTS WITH PRODIGIOSIN			TOTAL INOCULA		RESULTS WITH AUREOMYCIN*		
	Bact. ($\times 10^7$)	Amebae ($\times 10^3$)	Amebicidal Conc. (μgm/ml.)	Bact. Pop. ($\times 10^7$)	pH	Bact. ($\times 10^7$)	Amebae ($\times 10^3$)	Amebicidal Conc. (μgm/ml.)	Bact. Pop. ($\times 10^7$)	pH
Endamoeba histolytica:										
NRS + mixed flora						13	300+	>200	0.003	7.4
NRS + *A. aerogenes*	600	250	2.5	740	7.2	315	260	200	1.0	7.4
Endamoeba coli:										
So strain	340	200	20	1460	7.0	1360	215	>200	0.6	7.2
Jo strain	950	310	4	800	6.0	685	310	>200	35.0	6.5
Dientamoeba fragilis:										
Bi strain	10	330	4	1280	7.1	24	330	100	0.5	7.4
R strain	145	330	2–4	1140	7.1	66	330	40	140.0	7.3
Endolimax nana:										
T strain	18	320	40	225	7.0	10	320	100	14.0	7.3
Th strain	200	300	20	635	6.8	130	355	100	0.7	7.0

* The data are for the series under aerobic incubation, in which bacterial viability was greater than in the anaerobic series.

falls far short of the 25-fold increase noted by Bradin and Hansen. This factor will be re-examined in the DISCUSSION. Moreover, the validity of the recorded endpoints is open to serious question in view of the distinctly bactericidal action at the high concentrations required to eliminate the amebae. This will become further evident in the following section.

After the present work had been substantially completed, two new antibiotics, terramycin and fumagillin, were introduced elsewhere as promising amebicides. It was decided to test these agents[4] with the technique developed previously, but time permitted only relatively few comparative experiments on *E. histolytica*

[4] The sample of terramycin was provided through the kindness of Dr. W. Alan Wright of Chas. Pfizer & Co.; the sample of fumagillin was provided through the kindness of John S. Evans of The Upjohn Company.

and *E. coli*.[5] The results are shown in Table 5, with the values representing six experimental series for *E. histolytica* and three series for *E. coli*.

The data disclose that terramycin acts similarly to aureomycin, as might be expected from their close chemical relationship (Pepinsky and Watanabe, 1952). Fumagillin, on the other hand, exhibited an astounding amebicidal activity, of the same order as originally described by McCowen *et al.* (1951) and confirmed by Hrenoff and Nakamura (1951). It is of special interest that fumagillin also proved highly effective against *E. coli*, in view of the recent report by Anderson *et al.* (1952) that "Since the action of this antibiotic against nonpathogenic intestinal protozoa is not significant, one should be certain to distinguish between *E. histolytica* and *E. coli* during follow-up examinations." The present findings suggest further study before accepting that opinion.

In any event it seems quite clear that a potent amebicide has appeared with the advent of fumagillin. One striking feature is its essential inactivity against bacteria. Since the bacterial flora plays an important role in the total syndrome

TABLE 5

Comparative amebicidal activity of TERRAMYCIN *and* FUMAGILLIN *against* Endamoeba histolytica *and* E. coli

STRAINS OF AMEBAE	AMEBIC INOCULUM ($\times 10^3$)	RESULTS WITH TERRAMYCIN			AMEBIC INOCULUM ($\times 10^3$)	RESULTS WITH FUMAGILLIN		
		Amebicidal Conc. (μgm/ml.)	Bact. Pop. ($\times 10^7$)	pH		Amebicidal Conc. (μgm/ml.)	Bact. Pop. ($\times 10^7$)	pH
Endamoeba histolytica (UC + mixed flora)	230	133	10	7.4	250	0.013	>400	7.5
E. coli (So + mixed flora)	250	100–200	20	7.3	260	0.033	>500	7.2

of intestinal amebiasis, it should prove instructive to compare the therapeutic effect of fumagillin alone with that of combined fumagillin and an antibacterial agent like aureomycin or terramycin.

2. *Relation of oxidation-reduction potentials to survival of amebae and bacterial flora.* Oxidation-reduction (O/R) potentials of a culture medium represent one of the indices of its suitability for promoting growth, especially in the case of parasitic forms requiring low oxygen tension. These O/R potentials are usually expressed as E_h in millivolts, with low (more negative) potentials representing zones of reducing activity, and high (more positive) potentials indicating oxidized states less suitable for anaerobic organisms. Chang (1946) had reported that *E. histolytica* proliferates only under strongly reducing conditions, which originate through bacterial metabolism in culture media. In this laboratory (Balamuth and Wieboldt, 1951) a correlation was established between reducing activity of some individual bacteria and their relative capacity for supporting amebic growth. Of greater pertinence to the present study, Bradin and Hansen (*loc. cit.*) have shown convincingly that bacteriostatic chemical agents are not compatible

[5] The technical assistance of Duncan McCarthy is acknowledged in this phase of the work.

with survival of amebae, the sequence being: bacterial inhibition → more positive E_h → death of amebae. Bradin and Hansen also reported that anaerobic incubation shifted the amebicidal endpoints of such agents to higher concentrations by maintaining lower O/R potentials in test media.

The present study afforded a good opportunity to correlate O/R potentials with the data on bacterial survival at amebicidal levels of the various agents. Potential-time curves were consequently traced for all series of agents, although

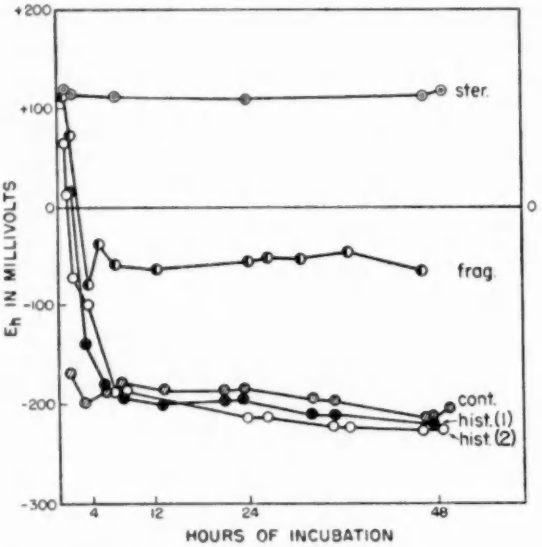

FIG. 1. Changes in oxidation-reduction potential of cultures of intestinal amebae containing amebicidal concentrations of emetine hydrochloride.

ster.: sterile test medium (liver-eggyolk infusion)

cont.: normal culture of *E. histolytica* (NRS + *Aerobacter aerogenes*)

hist. (1): NRS + *A. aerogenes* at 0.5 μgm/ml

hist. (2): NRS + flora at 4 μgm/ml

frag.: *Dientamoeba fragilis* (Bi) at 100 μgm/ml

the data for emetine, prodigiosin, and aureomycin may serve to illustrate the major relationships uncovered.

In reference to emetine, it was shown above that bacteria were not inhibited at amebicidal concentrations; hence agreement might be expected between O/R potentials of experimentals and controls. Figure 1 demonstrates this strikingly for both cultures of *E. histolytica*. It was observed early in our study that some strains (*e.g.*, the *Bi* strain of *Dientamoeba*) yielded consistently more positive potentials in normal cultures, doubtless through influence of the flora, but it was found in these cases that good amebic growth could be obtained below −50 mv. The curve for *Dientamoeba* illustrates this variation in intrinsic reducing

activity (see also Figure 2), since it is clear from Table 1 that bacterial viability was excellent.

A case similar to emetine is presented by prodigiosin (Figure 2). Here also negative O/R potentials were generated, as one should expect in the absence of bacteriostasis.

The critical test is offered by the other antibiotic, aureomycin. The curves in Figure 3 lend striking support to the view that O/R potentials reflect bacterial activity. The marked bacterial inhibition as expressed by viable populations (Table 4) was clearly accompanied by inability to achieve reducing conditions.

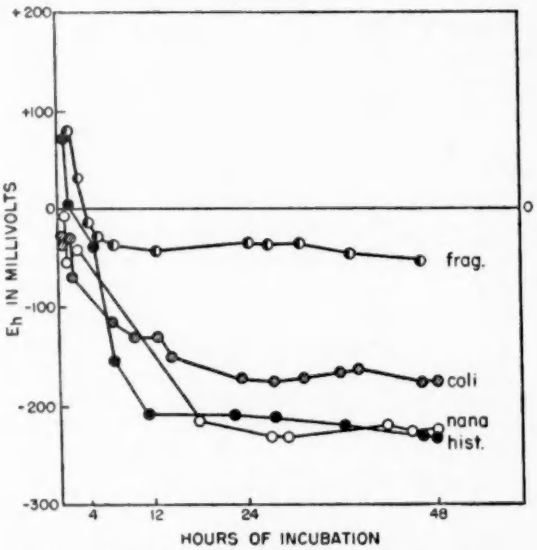

FIG. 2. Changes in oxidation-reduction potential of cultures of intestinal amebae containing amebicidal concentrations of prodigiosin.
hist.: NRS + *A. aerogenes* at 5 μgm/ml **coli:** *E. coli* (Jo) at 4 μgm/ml
nana: *Endolimax nana* (Th) at 20 μgm/ml **frag:** *D. fragilis* (Bi) at 4 μgm/ml

The two pairs of curves show, furthermore, that the O/R potentials varied directly with the concentration of aureomycin as well as with survival of the test amebae.

3. *Influence of the relative amount of inoculum on amebicidal endpoints.* It has been noted above that many variables operate in current *in vitro* testing programs. One of the most obvious is the inoculum itself in relation to the volume of test medium. In our early experiments no special attempt was made to control the numbers of amebae and bacteria, but only to record them. Under these conditions it was noted that instead of sharp endpoints fairly wide zones of amebicidal action were being obtained (*viz.*, up to fourfold differences in drug concentration for given combinations). The necessity of controlling this variable response led

to analysis of the influence of the inoculum on fixing specific endpoints. No correlation could be found between the size of the bacterial inoculum and viability,

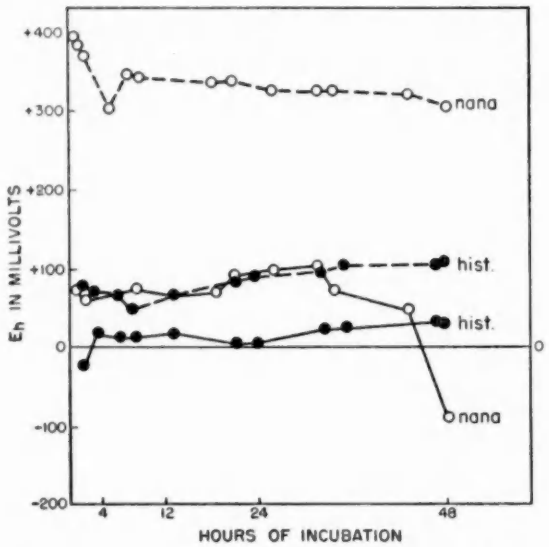

FIG. 3. Changes in oxidation-reduction potential of cultures of intestinal amebae containing threshold levels of aureomycin hydrochloride.

hist. (solid): NRS + *A. aerogenes* at 100 μgm/ml (survival)

hist. (dotted): NRS + *A. aerogenes* at 200 μgm/ml (death)

nana (solid): *Endolimax nana* (T) at 40 μgm/ml (survival)

nana (dotted): *E. nana* (T) at 100 μgm/ml (death)

TABLE 6

Influence of the relative amount of amebic inoculum on survival of intestinal amebae in threshold levels of amebicidal agents

STRAINS OF AMEBAE	AGENT + ENDPOINT	AMEBIC INOCULA ($\times 10^3$)	RESULT
	μgm/ml.		
Dientamoeba (Bi)	Emetine 100	330	Death
		1040	Survival
E. histolytica (NRS + *A. aerogenes*)	Aureomycin 100	123	Death
		260	Survival
E. coli (Jo)	Aureomycin 200	150	Death
		310	Survival
E. coli (Jo)	Prodigiosin 2	70	Death
		200	Survival

even though in some cases there was a hundred-fold difference in range of inoculum. This was not unexpected, especially for nonantibacterial agents, in view of the short generation time and the duration of the experiments, which combined

to yield final bacterial populations quite independent of the range of inocula used in the present experiments.

On the other hand, the size of the amebic inoculum definitely influenced the endpoint. Special experiments were subsequently performed to investigate the relationship and Table 6 shows some clearcut cases in which endpoints were shifted through adjustment of inocula. This finding is perhaps not surprising when it is recalled that, by definition, an endpoint requires a "100 per cent kill", but it emphasizes the importance of recording and controlling the inocula if experiments are to be subject to repetition.

DISCUSSION

The primary objective of the present study was to seek evidence of differential susceptibility of the various species of amebae. The possibility existed that pathogenic and nonpathogenic types might react differently, establishing the basis for a "physiological" classification.[6] Emetine has offered the only definite lead in this direction and the very fact that it was unique among the diverse classes of agents tested suggests the desirability of more intensive study of the mechanism of action of this drug. To the best of our knowledge, no *in vitro* evidence has been offered of differential species susceptibility to any other amebicidal agent. Stewart (1949) has made a preliminary attack on this phase of the problem in *E. histolytica* by describing morphological changes and altered growth rates at different concentrations of emetine. He concluded that emetine probably acts by specific interference with still unknown cytoplasmic reactions prior to fission. Dobell and his colleagues originally described the differential susceptibility of *E. histolytica* and *E. coli* to emetine, although the endpoints reported by them were closer together than in the present study. Thus Laidlaw, Dobell and Bishop (1928) discovered an endpoint for *E. histolytica* at 0.2 μgm. per ml. of emetine in four-day exposures; while Bishop (1929) used the same technique on *E. coli* to record an endpoint of 3.3 μgm. per ml. They also reported that the biological activity of emetine varied directly with increasing pH, and decreased markedly in diphasic media through absorption of the drug by the coagulated slant. We have recently obtained striking confirmation of the pH effect with emetine and certain related compounds (Balamuth and Lasslo, 1952). The lack of agreement on specific endpoints perhaps is not surprising in view of differences in testing conditions.

The relative standing of the agents used in the present study is quite clearcut and uniform with the exception just noted. Prodigiosin and carbarsone oxide displayed the greatest average activity against the complex of species, followed respectively by C. C. no. 914, emetine, Vioform, and aureomycin. On the other hand, emetine, prodigiosin, and carbarsone oxide ranked highest in acting specifically against *E. histolytica*. There are possible clinical applications of these find-

[6] The only human intestinal species not used was *Iodamoeba bütschlii*. Cultures of this form have proved difficult to establish owing to its low incidence and to difficulty in obtaining fecal specimens free of other protozoa. According to some authorities (Chandler, 1949) the normal host of this species in temperate climates is the pig, with a low incidence in the human.

ings in view of the prevalent attitude that *Dientamoeba* is a pathogen (Chandler, *loc. cit.*) and may require therapy (Anderson *et al.*, 1948). Indeed, it is well known that many physicians routinely prescribe treatment for any species discovered in symptomatic patients. Systemic toxicity has been reported for carbarsone oxide and prodigiosin, while the ineffectiveness of tolerated doses of emetine in intestinal amebiasis is well known. This would leave the dithio derivative of carbarsone oxide (C. C. no. 914)[7] as a highly promising broad-spectrum agent, since it has been reported that the substitution of —SH groups for oxygen markedly detoxifies carbarsone oxide.

The question of agreement in amebicidal endpoints between the present work and other studies must necessarily be restricted to the case of *E. histolytica*. It is apparent that the effects of any given agent extend across a broad range from inactivity through amebistasis to increasing amebicidal activity. In reality, therefore, an *amebicidal zone* might provide a more accurate picture than an arbitrary endpoint based upon a "100 per cent kill", and this must be considered in comparing specific endpoints reported by different laboratories. The most extensive data have emanated from Anderson's laboratory, as exemplified by Bradin and Hansen's use (*loc. cit.*) of monobacterial cultures containing the *Clostridium*-like "organism *t*". Under anaerobic incubation, which they considered more reliable, they reported the following endpoints (in μgm. per ml.): emetine, 10; Vioform, 20–100; carbarsone oxide, 20; C. C. no. 914, 50; prodigiosin, 10; aureomycin, 50. Thompson *et al.* (1950) obtained the following endpoints with cultures containing a mixed flora; emetine, 4.5; Vioform, 15; prodigiosin, 4; aureomycin, 188. Several workers have used monoxenic cultures containing *Trypanosoma cruzi* since Phillips (1950) introduced the combination. Thus Nakamura and Anderson (1951) fixed endpoints for the following agents: emetine, 10; Vioform, 40–50; C. C. no. 914, 33–50; aureomycin, 70–100; while Bradner and Rawson (1951) reported the following: emetine, 30; Vioform, 125; aureomycin, 60. Phillips (1951) employed single-ameba inocula in micro-tubes with a number of agents, including emetine, <5 and aureomycin, 100–250. The examples just cited disclose wide variability between laboratories which is not lessened by resort to monobacterial cultures or to the use of a protozoan associate with *E. histolytica*. All the quoted studies made use of fluid media; many other workers have used diphasic media containing slants of coagulated egg, but it has been shown conclusively that under these conditions drug activity is lessened by its selective removal from the fluid component (Laidlaw *et al.*, *loc. cit.*; Anderson, Johnstone and Hansen, 1947).

In conclusion, some general observations would seem to be in order regarding the status of *in vitro* testing techniques for intestinal amebae. There is no question that a standardized technique is strongly needed and long overdue in the interest of accuracy and repeatability of claims. This advance has been impeded by our present inability to maintain these parasites in pure culture as well as by ignorance of other requirements existing in their complex environment. In the absence of this knowledge it is still possible to recognize at least three classes

[7] This compound is now being distributed by Eli Lilly & Co. under the name of *Thiocarbarsone*.

of variables which are subject to control but which await definitive experimental analysis.

These variables involve respectively the inoculum, conditions in the test medium and criteria surrounding the amebicidal endpoint. The inoculum is a composite of amebae and associated organisms. In regard to the amebae, critical control is desirable in pooling operations (*cf.* MATERIALS AND METHODS), and in regulating the size and age of inocula. Many published studies (*e.g.*, even that of Dobell, *loc. cit.*) have not referred to numbers of amebae, while others (*e.g.*, Anderson's group, and Thompson *et al.*, 1950) have used up to 150,000 in 5 ml. total volume. Since the associates must support amebic growth while being subjected to drug action, their role is clearly important. It is of interest that monobacterial cultures tend to be somewhat more susceptible to drugs (*cf.* also Jones, 1950), suggesting that a mixed flora may offer greater protection even though it obviously introduces a more complex environment. The advantage claimed for *Trypanosoma cruzi* as an associate was its relative insusceptibility to drugs; in practice, however, the results have agreed closely with those involving bacteria and the testing technique is more complicated.

The general testing conditions include the medium itself, the type of incubation and the duration of exposure to drugs. A good medium should be monophasic and well buffered, should support good growth of amebae and its components should not interfere with the action of drugs. Many English workers have used a buffered serum-saline medium since the early work of Laidlaw *et al.* (*loc. cit.*), even though stock cutures usually cannot be routinely maintained in it. From the data submitted by Stewart (*loc. cit.*) a fivefold increase in amebae, reaching 50 per cu. mm., was noted in this medium; this compares with a thirtyfold increase, reaching 140 per cu. mm., recorded by Balamuth and Wieboldt (*loc. cit.*) in aqueous eggyolk infusion. In both cases polybacterial cultures were involved, although the latter medium as used in drug studies contains added liver extract and supports considerably larger populations. Bradin and Hansen (*loc. cit.*) have emphasized the value of anaerobic incubation in cases of bacterial inhibition. Our own experience suggests that anaerobic incubation should be employed with caution since the use of vaspar seals may lower the pH, depressing the activity of drugs like emetine, and at the same time may inhibit growth of aerobic bacteria like *Esch. coli* (Balamuth and Lasslo, *loc. cit.*).

The reliability of endpoints rests not only upon the above variables, but also upon criteria for establishing the viability of amebae and associates, as well as a suitably reduced level of the medium. Some pertinent data have been submitted in the present work. Nakamura and Anderson (1951) have recently measured the oxygen uptake of the associated *T. cruzi* in drug tests as an additional evidence of viability; this approach may offer a useful supplement to criteria of differential drug action.

SUMMARY

A series of six agents was tested *in vitro* against *Endamoeba histolytica, E. coli, Dientamoeba fragilis*, and *Endolimax nana*. The agents included emetine, Vioform,

carbarsone oxide, a dithio derivative of carbarsone oxide known as C. C. no. 914, prodigiosin and aureomycin.

Emetine alone exhibited a distinctly differential amebicidal action, *E. histolytica* proving at least twenty-five times more susceptible than any other species. Monobacterial cultures tended to be more susceptible than those containing a mixed flora.

The broadest activity spectra against all species were exhibited by prodigiosin, carbarsone oxide and C. C. no. 914. Contrary to several published reports, aureomycin was found to have relatively little direct amebicidal activity.

From a consideration of the many variables operating during *in vitro* drug tests, it is concluded that careful control is required of such factors as the inocula, conditions existing in the test medium and criteria for determining amebicidal endpoints.

Supplementary tests of terramycin and fumagillin against *E. histolytica* and *E. coli* corroborated the claim that fumagillin is the most potent amebicide *in vitro* yet discovered.

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DRUG EFFECTS ON THE METABOLISM OF *ENDAMOEBA HISTOLYTICA*; *IN VITRO* AND *IN VIVO* TESTS OF SYNERGISM

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INTRODUCTION

A variety of chemical types have been shown to inhibit *Endamoeba histolytica in vitro*, but their effects on the metabolic activity of the ameba is unknown. In fact, little evidence is available as to the mechanism of action of these agents. No truly satisfactory method is available to measure the metabolism of amebas chiefly because of their slow rate of growth as compared with that of associated bacteria. Even though the rate is slow some detectable metabolic activity of the ameba does occur. Chemical analyses of cultures of *E. histolytica* associated with a "streptobacillus" by Karlsson have indicated that there may be some organic acids produced by the amebas. This observation was based on culture studies of *E. histolytica* which produced acids more volatile and of a different composition than those produced by the "streptobacillus" alone in the same medium. The present report describes a method of measuring manometrically the acid (and gas) production by *E. histolytica* and the effect of certain drugs on its metabolic activity. Some evidence of the synergistic action of drugs against *E. histolytica* is presented also both *in vitro* and *in vivo* following an earlier preliminary report (Nakamura *et al.*, 1952). These studies may suggest possible modes of action of some of the amebicides currently of interest.

IN VITRO TESTS UTILIZING MANOMETRIC TECHNICS

Materials and methods

The organisms used *in vitro* were *E. histolytica* associated in cultures with a "streptobacillus" obtained from Dr. C. W. Rees, National Institutes of Health, Bethesda, Md. This culture was grown on a proteose-peptone-liver extract medium of Hansen (1950). Earlier studies have shown that 20,000 units of penicillin G effectively inhibits the "streptobacillus" required in cultures of *E. histolytica*. (Nakamura *et al.*, unpubl.) After the cultures have been incubated at 37°C. for three days, 6 mgm. of penicillin G per tube (4 ml.) were added and the tube sealed anaerobically with a pyrogallic acid seal. The technic for sealing consisted of clipping the top of the cotton plug and pushing it deeper into the tube and plugging with nonabsorbent cotton as well. To the absorbent cotton capping

[1] With the technical assistance of Mrs. Betty Mythen and Miss Dorothy Swanman. Consultation with Drs. Jon L. Karlsson and Blair M. Bennett is also acknowledged with gratitude, in certain technical aspects of this study.

[2] Supported, in part, by the National Institutes of Health, United States Public Health Service, Bethesda, Md.

were added 0.4 ml. of 20 per cent pyrogallic acid and 0.7 ml. of 20 per cent sodium carbonate. The tubes were then sealed tightly with a rubber stopper. After 24-hours incubation, the bacteria were inhibited but the amebas continued to multiply. The anaerobic seal prevented oxidation and thus provided an environment favorable for growth of the amebas. The tubes were re-incubated for 24 hours after the antibiotic had been added.

Amebas from ten culture tubes were harvested and concentrated by three washings and centrifugations with normal saline at 750 to 800 r.p.m. The supernatant fluid from the final centrifugation was set aside as the source of residual bacteria which would measure the metabolic activity contributed by the bacterial associate alone. The final centrifuged preparation was resuspended in 10 ml. of saline and the number of amebas was counted in a hemocytometer. Dilution or recentrifugation brought the count of amebas to 2,000,000 per ml. or more. During the various phases of handling the amebas, 5 mgm. of cysteine was added in order to maintain an optimal reduced environment. This addition, however, might cause some spontaneous evolution of gas, but was accounted for in the control tubes. It was found that the gas evolved did not contribute significantly to the differences of amebic activity noted.

To each Warburg flask of 10 ml. capacity were added 2.17 ml. of an amebic suspension, 0.3 ml. of 0.15 M sodium bicarbonate (this concentration became 0.015 M in a final fluid volume of 3.0 ml.) and the mixture was maintained at pH 7.2 in a 5 per cent carbon dioxide atmosphere. At this time, 10 mgm. of penicillin G, 5 mgm. of streptomycin, 5 mgm. of cysteine HCl, and 10 mgm. of glucose were added. Drugs were then added for amebicidal testing in appropriate concentrations. Distilled water was added to make each flask up to a fluid volume of 3.0 ml. To the bacteria control culture tube was added 2.17 ml. of supernatant fluid from the centrifuge tube aliquot after the third washing and recentrifugation of amebas, instead of the amebic suspension. Since this preparation did not contain any amebas but only the associated bacteria, the flask acted as a control for the small amount of bacterial metabolic activity which may have been present.

All of the flasks were placed in an incubator at 37°C. for 35 minutes. They were then flooded with a 5 per cent carbon dioxide-95 per cent nitrogen gas mixture for 5 minutes to evacuate the oxygen so as to obtain an anaerobic environment. The vessels were equilibrated for a period of 10 minutes, readjusted, then equilibrated again for 5 minutes. This procedure made possible determination of an initial control value one hour after the organisms had been placed in contact with the antibiotics added to inhibit the associated bacteria, and to allow the amebicides to act against the amebas directly. Penicillin and streptomycin utilized to inhibit the "streptobacillus" were used in concentrations that were found to be nontoxic to the amebas (Bradin *et al.*, 1951). Control flasks of amebas contained no test amebicides.

Manometric readings were taken every 10 minutes for a total of 70 minutes. The manometers were agitated at a rate of 20 shakes per minute. The activity was measured as c.mm. of gas formation which included carbon dioxide and acid

production. However, it is less likely that carbon dioxide is produced in appreciable amounts by the amebas since *E. histolytica* in cultures with *Trypanosoma cruzi* which have been sealed with petrolatum provide no visible evidence of gas formation after three to four days of incubation. Since starch is a component of the medium in the "streptobacillus" culture, however, the trypanosome associate culture is not a completely valid control. After the manometric determinations were completed, the flasks were removed and a drop of the suspension was examined microscopically to study the appearance of the amebas.

Results and discussion

Microscopic examination of the amebas at the end of the manometric studies showed that there was no apparent damage to the control or untreated amebas. These were actively motile, but in flasks containing amebicides, the amebas were immobilized, rounded and appeared "granular".

TABLE 1

Per cent inhibition of control ameba activity values by drugs at the end of 70 minutes in the Warburg manometer*

DRUG	CONCENTRATION	EXPERIMENT					MEAN
		#1	#2	#3	#4	#5	
Fumagillin	1/20,000	81.3%	97.4%	93.9%	90.0%	91.6%	90.8%
Terramycin	1/20,000	6.6	12.1	8.3	16.7	14.9	11.7
Aureomycin	1/20,000	86.5	88.0	82.4	85.7	84.4	85.4
Emetine	1/20,000	65.4	53.5	20.2	69.9	71.0	56.0
Thiocarbarsone	1/20,000	22.3	36.2	25.9	36.5	39.5	32.1
Fumagillin	1/80,000	8.7	0.0	2.8	0.0	11.7	4.6
Terramycin	1/80,000	7.3	20.0	12.7	20.8	10.1	14.2
Aureomycin	1/80,000	49.0	45.6	54.8	63.8	71.1	56.8

* The amount of acid ($+CO_2$) produced as measured by gas formation during the period of observation.

Table 1 shows the effect of various amebicidal agents on metabolic activity. The figures indicate the per cent inhibition, by test drugs at the end of the 70 minute observation period. These values were obtained by subtracting the total bacterial activity from the amebic control activity level. This final value indicated the activity of control amebas not exposed to the amebicides under study. The direct effect of these drugs on metabolism was expressed as the per cent inhibition of this control value.

It is noted in Table 1 that fumagillin appeared to have the greatest inhibitory action on the metabolism of the amebas, at a concentration of 1/20,000, followed by aureomycin, emetine, thiocarbarsone (C.C. 914), and terramycin at the same concentrations. Fumagillin at 1/80,000 showed little inhibitory effect for the short period of exposure, indicating a slower rate of activity than either aureomycin or terramycin.

When combinations of these drugs were used in the same test, marked increases

in the inhibitory effect were noted in several cases. Fumagillin (1/80,000) alone caused 4.6% inhibition of the amebas and thiocarbarsone (1/20,000) alone caused 32.1% inhibition, yet in combination there was a total inhibition of 61.6%, which was interpreted as synergism. A similar occurrence was seen in the case of fumagillin (1/80,000) which caused 4.6% inhibition alone, and terramycin (1/20,000) which caused 11.7% inhibition; yet the two drugs combined caused 97.2% inhibition, again indicating a synergistic mechanism. On the other hand, combinations of aureomycin (1/80,000) and terramycin (1/80,000) appeared to be approximately additive, as did emetine (1/20,000) and thiocarbarsone (1/20,000). Table 2 summarizes these results.

TABLE 2

Per cent inhibition of control ameba activity values by combinations of drugs at the end of 70 minutes in the Warburg manometer*

DRUG COMBINATION	CONCENTRATION	EXPERIMENT					MEAN
		#1	#2	#3	#4	#5	
Fumagillin + Thiocarbarsone	1/80,000 1/20,000	87.0%	53.5	61.9	63.7	72.1	61.6†
Fumagillin + Terramycin	1/80,000 1/20,000	100.0	100.0	96.0	98.0	92.2	97.2†
Fumagillin + Aureomycin	1/20,000 1/20,000	100.0	100.0	100.0	100.0	86.5	97.3
Aureomycin + Terramycin	1/80,000 1/80,000	68.1	65.3	75.0	65.8	88.0	72.6
Thiocarbarsone + Emetine	1/20,000 1/20,000	73.8	92.5	81.5	84.0	79.9	82.3

* The amount of acid ($+CO_2$) produced as measured by gas formation during the period of observation.

† Statistical evidence of synergism was obtained for these two combinations only, i.e., more than an additive effect.

Statistical analyses of the experimental data were performed by the arc sine transformation of per cents using Fisher and Yates' Table XII (1948) and then applying the Student t-test to determine the significant differences of the values compared. The analyses indicated that: fumagillin (1/80,000) and thiocarbarsone (1/20,000) used in combination exhibited synergistic action; fumagillin (1/80,000) and terramycin (1/20,000) used in combination exhibited synergistic action; aureomycin (1/80,000) and terramycin (1/80,000) used in combination did not show synergism but rather additive effects; thiocarbarsone (1/20,000) and emetine (1/20,000) used in combination did not show synergism but additive effects only.

The total time amebas were exposed to test agents was 130 minutes, i.e. 60 minutes before the first manometric reading and 70 minutes after, for continuous observation. Therefore, those drugs which showed slight initial action may have been too slow acting for this short period of observation. Other agents are believed not to have exhibited amebicidal action but had shown amebicidal action in pre-

vious *in vitro* drug tests by virtue of their primary antibacterial effect against the bacterial associate. Drugs such as fumagillin appeared to act directly, but at a slower rate than emetine, on this test and in previous studies. Emetine appeared to act immediately and directly but somewhat less effectively than fumagillin. Terramycin, in some tests, required more than 120 minutes to inhibit amebic activity. Such tests may demonstrate action by drugs not changed to more active derivatives by host metabolic processes.

The activity of fumagillin *in vitro* (on 48 hours exposure) has been shown by McCowen, *et al.* (1951) and subsequently (Hrenoff *et al.*, 1951), to be amebicidal at a range of from 1/8,000,000 to 1/131,000,000. In the earlier report (McCowen *et al.*, 1951; Anderson, 1951) it was suggested that fumagillin exhibited direct action as an amebicide. Although fumagillin was active in very dilute solutions, for the present studies concentrations of 1/20,000 to 1/80,000 were used in order to compare similar levels of various drugs and also because the exposure period was very short. One other reason for using higher concentrations than those found to be effective in 48-hour *in vitro* tests was that there were more than 2,000,000 amebas per ml. in each Warburg flask. In 48-hour cultures the amebas numbered from 100,000 to 300,000 per tube (4.0 ml.).

The method reported here of measuring acid and gas formation during amebic activity provides a technic for initiating studies of the anaerobic metabolism of *E. histolytica*. It provides a means of detecting early amebicidal effects before irreversible changes or death of the amebas occurs. This is an advantage in the elucidation of mechanism of drug action so far as rate, and direct vs. indirect amebicidal effects are concerned. However, it is realized that this method is not an ideal evaluating procedure since the results may be misleading as in the case of more slowly acting drugs. Furthermore, the influence of the bacterial associate cannot be fully weighed. Thus, drugs which are temporarily effecti ve in the lumen of the bowel and which may act indirectly by inhibiting the intestinal bacterial flora would not be appraised accurately by this procedure.

IN VIVO SYNERGISM IN MACAQUE AMEBIASIS

Synergistic action of two or more drugs against *E. histolytica* in macaque amebiasis had been suggested by earlier studies in this laboratory by Anderson and Anderson (1950) and by Hrenoff and Anderson (1950). The finding that synergism occurred *in vitro* between terramycin (1/20,000) and fumagillin (1/80,000) prompted additional trials in monkeys to determine whether or not *in vivo* synergistic action could be demonstrated.

Previous trials in monkeys with terramycin and fumagillin given singly to naturally infected animals indicated that each antibiotic could clear the majority of animals treated. The daily dose of each antibiotic in the present study was 12.5 mgm. per kilo given orally together over 10 consecutive days. This dose is equivalent to one-fourth of the smallest dose of each agent (50 mgm./Kg.) which was found to be effective when used alone (Hrenoff, 1951; Hrenoff *et al.*, 1951).

The procedure followed has been reported elsewhere (Anderson *et al.*, 1950; Hrenoff, 1951). Fixed fecal smears were stained with iron-hematoxylin and were

searched for parasites daily upon completion of the treatment. Table 3 summarizes the findings.

TABLE 3

Concurrent use of two "amebicidal" antibiotics given orally in ¼ the lowest effective dose given singly

MONKEY NO.	DOSE OF TERRAMYCIN HCl in mgm./Kg./day	DOSE OF FUMAGILLIN in mg./Kg./day	*E. histolytica*
1	12.5	12.5	Absent after 12 weeks
3	12.5	12.5	Absent after 12 weeks
4	12.5	12.5	Absent after 12 weeks
5	12.5	12.5	Absent after 12 weeks
6	12.5	12.5	Recurrence in 3 weeks

SUMMARY

1. An *in vitro* method is described for measuring the anaerobic metabolism of *E. histolytica* in terms of acid and CO_2 formation, utilizing manometric technics.

2. The effects of several agents on the metabolic activity of amebas have been studied. Synergism was observed when drug combinations were tested *in vitro*, notably with fumagillin and terramycin and with fumagillin and thiocarbarsone (C.C. 914).

3. Four of five naturally infected macaques were cleared over a 12 week period when ¼ the smallest effective oral dose of fumagillin and terramycin (each 12.5 mgm./Kg.) were given together daily over ten days.

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THE EFFECTS OF ERYTHROMYCIN (ILOTYCIN, LILLY) AGAINST CERTAIN PARASITIC ORGANISMS[1]

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In a previous publication, McGuire *et al.* (1952) have reported a new antibiotic, erythromycin, (Ilotycin, Lilly), isolated from a strain of *Streptomyces erythreus*. Preliminary studies with erythromycin indicated this antibiotic to be of low toxicity and to be active against gram-positive and some gram-negative bacteria, typhus rickettsiae, certain large viruses, and certain protozoa and helminths. The purpose of this communication is to supplement the chemotherapy data against parasitic organisms given in the original report.

IN VITRO TESTS

Effect against Endamoeba histolytica. Twofold serial dilutions of erythromycin were tested in cultures of *E. histolytica* (Strain NIH 200 with associated bacterial flora) in liquid egg-yolk infusion medium (Balamuth, 1946). Erythromycin test

TABLE 1

Comparative effectiveness of erythromycin, aureomycin, and terramycin against E. histolytica *in vitro*

ANTIBIOTIC	AMEBICIDAL DILUTIONS IN VITRO	
	Egg yolk infusion medium*	Shaffer-Frye medium†
Erythromycin	1:8,000	<1:2,000
Aureomycin	1:8,000	1:16,000
Terramycin	1:16,000	1:8,000

* Negative cultures at 48 hours were subcultured and incubated for an additional 48 hours at 37°C.

† Negative cultures at 72 hours were subcultured and incubated for an additional 72 hours at 37°C.

cultures and controls without antibiotic were incubated for forty-eight hours at 37°C. The endpoint of minimum effective dilution was determined by microscopic examinations. The amebicidal endpoint was substantiated by subcultures and an additional forty-eight hours incubation at 37°C.

In order to compare the activity of other antibiotics, aureomycin and terramycin were tested *in vitro* against *E. histolytica* under the same test conditions. Table 1 shows the amebicidal endpoints of erythromycin, aureomycin, and terramycin against *E. histolytica* with associated bacterial flora *in vitro*.

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Twofold serial dilutions of erythromycin, aureomycin and terramycin also were tested in cultures of *E. histolytica* (Strain NIH 200 without multiplying bacteria) in Shaffer-Frye medium (Shaffer *et al.*, 1948). Antibiotic test cultures and cultures without antibiotics were incubated for seventy-two hours at 37°C. Amebicidal endpoints were determined by microscopic examinations. The endpoint of each antibiotic was confirmed by subcultures and incubated for an additional seventy-two hours at 37°C. Results of tests using the Shaffer-Frye medium are summarized in Table 1.

As shown in Table 1, each of the antibiotics tested demonstrated some degree of amebicidal activity, when tested *in vitro* with associated bacterial flora or without multiplying bacteria, with the exception of erythromycin, which demonstrates greater activity against *E. histolytica* only when bacterial associates are present in culture.

IN VIVO TESTS

Twenty-one-day-old rats of the Wistar strain were preconditioned on a 90 per cent fish diet for forty-eight hours. Afterwards, approximately 50,000 to 100,000 cultivated cysts (Balamuth, 1951) of *E. histolytica* (Strain NIH 200) were inoculated orally (McCowen and Lawlis, 1950). The rats were given erythromycin, aureomycin, and terramycin orally twice daily for two days. Treated and control animals without treatment were sacrificed on the fifth day following the inoculation with amebae. The effect of the different antibiotics was determined by microscopic examination of the cecal content of each rat for the presence or absence of *E. histolytica*. Aureomycin and terramycin also were tested under the same conditions as erythromycin.

Table 2 shows the effect of erythromycin, aureomycin, and terramycin against *E. histolytica* infections in young rats. Under the test conditions, erythromycin is more active *in vivo*, milligram per kilogram of body weight, than the other antibiotics tested.

Effect against Borrelia novyi *infections in mice.* White mice weighing 17–19 Gms. were infected intraperitoneally with 0.1 ml. of a suspension of *B. novyi*-infected rat blood in a 2 per cent sodium citrate and 0.85 per cent saline solution. Approximately six hours after the mice were inoculated, treatment with erythromycin, terramycin, and penicillin-G, crystalline-potassium, U.S.P. was begun. A single dose of the antibiotics was given orally to each mouse in certain groups and intraperitoneally to others. Controls received no antibiotics. Approximately eighteen hours and again at forty-eight hours after treatment, tail blood of each mouse was examined for spirochetes, using a dark-field microscope.

Table 3 shows the effect of erythromycin, terramycin, and penicillin-G, crystalline-potassium, U.S.P. when tested against *B. novyi* infected mice. It is apparent that each antibiotic tested is effective against *B. novyi* when administered orally or parenterally to mice.

Effect against oxyurids in mice. Groups of eight white mice weighing 17–19 Gms., with two species of naturally occurring oxyurids, *Syphacia obvelata* and *Aspiculuris tetraptera*, were fed 0.5, 0.25, and 0.125 per cent concentrations of

erythromycin in a commercial laboratory mouse diet for seven days. Control mice received no antibiotic in their diet. Mice in the treated and untreated groups were not given any diet for approximately twenty-four hours before they

TABLE 2

Comparative effectiveness of erythromycin, aureomycin, and terramycin against E. histolytica *in rats*

ANTIBIOTIC	TOTAL ORAL DOSAGE MG./KG.	RATS CLEARED / RATS TESTED	NON-TREATED CONTROLS
Erythromycin	1,000	5/5	0/5
	500	17/20	4/26
	250	3/10	1/9
Terramycin	500	2/5	1/4
	250	2/5	1/4
Aureomycin	500	2/8	4/16
	250	1/5	4/16

TABLE 3

Comparative effectiveness of erythromycin, terramycin, and penicillin against B. novyi *infections in mice*

ANTIBIOTIC	ROUTE OF ADMINISTRATION	SINGLE DOSAGE MG./MOUSE	EFFECT IN MICE*
Erythromycin	oral	5.0	10/10
	oral	2.5	5/10
	oral	1.0	0/10
	i.p.	2.5	10/10
	i.p.	1.0	3/14
	i.p.	0.5	0/10
Terramycin	oral	5.0	8/8
	oral	1.0	0/4
	i.p.	0.5	8/8
	i.p.	0.1	2/8
Penicillin G Crystalline Potassium, U.S.P.	oral	1.5	4/4
	oral	1.0	6/8
	oral	0.5	0/4
	i.p.	1.0	5/8
	i.p.	0.5	8/20
	i.p.	0.2	0/4
Non-treated	—	—	0/46

* Numerator indicates number of mice cleared of spirochetes; denominator indicates number of mice tested.

i.p. indicates intraperitoneal route

were sacrificed. This period without food permitted the removal of most of the fecal debris from the cecum and large intestine and thereby facilitated the examination for oxyurids. The cecum and large intestine were removed from each mouse and the contents perfused with a solution of 0.85 per cent saline and 0.75 per cent sodium citrate under five pounds of compressed air. The perfusate

from the cecum and the large intestine of each mouse was collected in a petri dish and microscopic examinations were made for oxyurids. Comparative counts of each species of oxyurids in the treated and untreated groups were made to determine the efficacy of erythromycin against oxyurids in mice. Table 4 summarizes the effect of erythromycin against naturally occurring oxyurids in mice.

The reduction of worm burden in each group was found to be significant as shown by the standard error of the mean in Table 4. However, it is evident that erythromycin was more effective against *S. obvelata* than against *A. tetraptera*. It is also assumed that erythromycin is effective against mature oxyurids since the few worms recovered were primarily immature forms. Untreated mice were found to harbor mature as well as immature oxyurids.

Effect against toxoplasmosis in mice. In preliminary studies, erythromycin was observed to have effect against toxoplasma infections in mice when administered orally. Because of these earlier observations, groups of six white mice weighing

TABLE 4

Effect of erythromycin against oxyurids in mice

PER CENT DIET 7 DAYS	AVERAGE WORMS/MOUSE		MICE CLEARED OF *A. tetraptera*		MICE CLEARED OF *S. obvelata*	
	Treated	Control	Treated	Control	Treated	Control
0.5	1.62 ± .98*	186.2 ± 37.1	5/8†	0/8	8/8	0/8
0.25	6.62 ± 4.45	186.2 ± 37.1	5/8	0/8	8/8	0/8
0.125	11.87 ± 7.25	186.2 ± 37.1	3/8	0/8	6/8	0/8

* Standard error of the mean

† Numerator indicates number of mice cleared; denominator indicates number of mice tested

17–19 Gms. were inoculated intraperitoneally with 0.1 ml. of dilutions 10^{-1} to 10^{-6} of freshly harvested mouse peritoneal fluid containing the R.H. strain of toxoplasma. Such peritoneal fluids titrate approximately 10^{-6}. Each dilution was prepared in 0.85 per cent saline.

The day prior to inoculation the mice were fed a commercial laboratory mouse diet containing 1.0 per cent and 0.5 per cent concentrations of erythromycin. Antibiotic feedings were continued for a total of seven days, after which a diet without antibiotic was fed for the remainder of the test period.

For the mice used in the test groups the LD_{50} titer calculations for the tenth day were made according to Bliss (1935). LD_{50} figures were computed using a combined slope.

Table 5 summarizes the effect of erythromycin against experimental toxoplasmosis in mice. It is evident from the data shown in this table that erythromycin has definite protective effect against toxoplasma in mice. The effect was proportional to the concentration of erythromycin in the diet of the animals. The 1.0 per cent diet of erythromycin effected a 10,000-fold protection when the antibiotic-treated mice were compared to nontreated control mice. Similarly, the 0.5 per cent erythromycin diet effected approximately a 1,000-fold protection.

Further observations of mice surviving the ten day test period were made. Deaths occurred in almost all treated mice between the tenth and eighteenth days. Mice which received the lowest dilution of the toxoplasma inoculum tended to die first, while those mice receiving the 1.0 per cent concentration of erythro-

TABLE 5

Effect of erythromycin against toxoplasmosis in mice

CONCENTRATION IN DIET (PER CENT) 7 DAYS	PROPORTION OF MICE DYING AT DILUTIONS:						LD50 LOG TITER ON 10TH DAY
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Erythromycin 1.0	6/6	1/6	0/6	0/6	0/6	0/6	1.7
Erythromycin 0.5	6/6	5/6	3/6	1/6*	1/6†	0/6	3.2
Non-treated controls	6/6	6/6	6/6	6/6	6/6	3/6	6.1

* Two mice were protected for 35 days before autopsy

† One mouse was protected for 35 days before autopsy

TABLE 6

Effects of erythromycin, aureomycin, and terramycin against T. equiperdum *and* T. gambiense

Trypanosoma SPECIES	ANTIBIOTIC AND ORAL DOSAGE MG./MOUSE	NUMBER OF TREATMENTS	DEATHS AFTER INOCULATION				NO. MICE SURVIVING 30 DAYS
			1–2 days	3–5 days	6–15 days	16–30 days	
T. equiperdum	Erythromycin						
	40 mg.	5	0	1	0	0	10
	20 mg.	4	0	0	1	8	1
	10 mg.	3	0	10	—	—	0
	Terramycin						
	40 mg.	2*	0	4	—	—	0
	Aureomycin						
	40 mg.	2*	0	4	—	—	0
Non-treated controls	—	—	0	15	—	—	0
T. gambiense	Erythromycin						
	40 mg.	5	0	2	8	1	0
	Terramycin						
	40 mg.	2*	0	4	—	—	0
	Aureomycin						
	40 mg.	2*	0	4	—	—	0
Non-treated controls	—	—	0	15	—	—	0

* Mice died before additional treatments could be given

mycin in the diet died several days later. A few mice in the 10^{-4}, 10^{-5}, and 10^{-6} dilutions group, which fed on the 0.5 per cent antibiotic diet, survived thirty-five days. Five mice out of six in the 10^{-6} dilution group survived thirty-five days after feeding on the 1.0 per cent erythromycin diet for seven days.

The mice surviving the 10^{-4} and 10^{-5} dilutions of toxoplasma were sacrificed after thirty-five days. Ten per cent suspensions of the brain, liver, and spleen

were prepared in 0.85 per cent saline. Three/one-hundredths ml. of the brain suspensions were inoculated intracerebrally into four normal mice for each dilution. Twenty-five/one-hundredths ml. of the liver and spleen suspensions were inoculated intraperitoneally into four normal mice for each dilution. No deaths or symptoms of toxoplasmosis were observed in these mice after twenty days. This was further evidence that erythromycin had prevented infection or eradicated toxoplasma in a few mice.

Effect against trypanosomiasis in mice. Groups of white mice weighing 17–19 Gms. were inoculated intraperitoneally with one million organisms of *Trypanosoma equiperdum* and *Trypanosoma gambiense* prepared in 0.85 per cent saline and 2.0 per cent sodium citrate solution. Six hours after inoculation, mice were given oral administrations of erythromycin daily for five days. Control groups were inoculated but no antibiotic treatment was given.

Table 6 summarizes the effect of erythromycin against *T. equiperdum* and *T. gambiense*. Aureomycin and terramycin were found to be ineffective against each species of *Trypanosoma*, while erythromycin was effective against *T. equiperdum* when given orally. Since aureomycin and terramycin did not demonstrate any protection, it was only possible to give two doses of these antibiotics before the infected mice died, while erythromycin-treated mice received five doses.

Erythromycin was found to be ineffective when administered orally against *T. cruzi* in C3H mice at the dosage effective for *T. equiperdum*.

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SUMMARY

Results of experimental studies with a new antibiotic, erythromycin (Ilotycin), tested against *Endamoeba histolytica*, *Borrelia novyi*, toxoplasma, oxyurid infections in mice and trypanosomiasis in mice, showed that:

1. *In vitro*, erythromycin, terramycin, and aureomycin demonstrated similar amebicidal activity against *E. histolytica* when bacterial associates were present, but in the absence of multiplying bacteria, erythromycin was least active, and when given orally to young rats infected with *E. histolytica* was found to be more effective per kilogram of body weight than terramycin or aureomycin.

2. Erythromycin was found to be effective against *B. novyi* infected mice when administered orally or parenterally.

3. Erythromycin fed in the diet of mice infected with oxyurids was found to reduce the worm burden significantly and to remove all *Syphacia obvelata*.

4. Erythromycin was found to have protective effect against toxoplasma in mice and in some mice prevented infection.

5. Erythromycin prevented death when given orally to mice infected with

Trypanosoma equiperdum. No effect was observed against *T. gambiense* or *T. cruzi, in vivo*.

Further investigations are necessary to determine whether erythromycin (Ilotycin) may be indicated in the treatment of amebiasis, spirochaetal infections, oxyuriasis, toxoplasmosis, and certain species of *Trypanosoma* infections.

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COMPARISON OF STARCHES USED IN THE CULTURE OF ENDAMOEBA HISTOLYTICA

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Dobell and Laidlaw (1926) observed that starch grains added to media greatly enhanced growth of *Endamoeba histolytica*. These investigators found that starches from rye, maize, buckwheat and rice all were satisfactory for this type of culture, but that rice starch, because of the smallness of its particles, was best. Since that time rice starch in some form has been added to the liquid phase of almost all newly developed media. Cleveland and Collier (1930) found that rice flour or ground unpolished rice was better than relatively pure rice starch.

In this laboratory, a cultural comparison of rice starch (A) with a rice powder (B) confirmed the observations reported by Cleveland and Collier. The NRS strain of ameba was grown under standard conditions in a filtrate of heat-coagulated serum as a liquid medium to which was added 50 milligram amounts of the test products. Counts of the yields were made after 48 hours to determine the relative degree of growth stimulation of each of the rice products (Hallman, Michaelson, and De Lamater, 1950). The results of this experiment are found in Table 1.

TABLE 1

	TROPHOZOITES PER ML.
Rice starch (A)	325,000
Rice powder (B)	650,000

The present report is concerned with the analysis of these substances. This work was undertaken in the hope that information might be obtained which would shed light on the growth requirements of *E. histolytica*.

MATERIALS AND METHODS

Two plant products were analyzed: (A) a finely milled rice starch, microscopically free of extraneous material, made for use as face powder and obtained through the courtesy of Mr. Pierre Depland of Roger and Gallet Company, New York, and (B) Bacto Rice Powder, a powdered rice grain (flour).

Quantitative determinations were made on these substances for carbohydrate, lipid, nitrogenous constituents, vitamins, and total inorganic constituents.

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All of these rice products were heat sterilized before analysis so that analysis would indicate as closely as possible the content of the material added to cultures. Starch (A) was routinely sterilized with dry heat at 180°C. for 60 minutes. Rice powder (B) was sterilized at 150°C. with dry heat for 90 minutes in order to avoid the marked discoloration of this substance which occurs at higher temperature. This temperature proved adequate to insure complete sterilization. All determinations were made in duplicate.

Carbohydrate

Total carbohydrate determination. One gram samples of the test starches were prepared by autoclaving for 10 minutes in 50% H_2SO_4. The total carbohydrate content of each test starch was determined by adaptation of the orcinal reaction (Sørensen and Hougaard, 1933). The percentage of total carbohydrate found in each product is given in column 1 of Table 2.

TABLE 2

Milligrams per gram

PRODUCT	TOTAL CARBOHYDRATE	WATER SOLUBLE MATERIAL	WATER SOLUBLE CARBOHYDRATE IN WATER EXTRACTED MATERIAL
Rice starch (A)	870	11.1	5.4
Rice powder (B)	810	21.9	9.3

Soluble carbohydrate (Hallman *et al.*, 1950). One gram samples of each of the starches were suspended in cold distilled water. The tubes were shaken mechanically for five minutes, centrifuged and filtered. An aliquot of the filtrate was dried to constant weight. The total soluble substance was then calculated. The amount of water soluble carbohydrate was then determined on the remainder of the filtrate. The short extraction period was used to determine how much carbohydrate was available to the ameba immediately after the addition of starch to the medium. The results are shown in column 2 of Table 2.

Lipids

Total lipid was determined by Soxlet extraction of 1 gram samples of the rice powder and starches with a mixture of 50% ether and 50% acetone for a 24 hour period. The ether-acetone mixture was then filtered, evaporated to dryness on a water bath, cooled in a desiccator and weighed to constant weight. This gave the total lipid content of the sample.

The lipid material was then saponified with alcoholic potassium hydroxide. The resulting solution was adjusted to pH 2 with 6 N hydrochloric acid and extracted with ether. The ether was washed with water, evaporated and dried. This procedure gave the fatty acid content of the material extracted. The difference between the fatty acid value and the total is recorded as nonsaponifiable material. The results of the lipid determinations are found in Table 3.

Inorganic constituents

Ash and moisture determinations were also made on rice powder and the rice starch used (Association of Official Agricultural Chemists, 1945). The results are found in Table 4.

Nitrogenous constituents

Total nitrogen was determined by a macro-Kjeldahl method which was found to be most suitable for use with one and two gram samples. Nonprotein nitrogen is recorded as the difference between total amino acid nitrogen and total nitrogen.

TABLE 3

Percentage by weight

PRODUCT	TOTAL LIPID	FATTY ACID	NONSAPONIFIABLE
Rice starch (A)	0.17	0.16	0.01
Rice powder (B)	1.13	1.01	0.12

TABLE 4

Percentage by weight

PRODUCT	ASH	MOISTURE
Rice starch (A)	0.5	8.8
Rice powder (B)	0.9	8.0

TABLE 5

Milligrams per 100 grams

PRODUCT	TOTAL NITROGEN	TOTAL AMINO ACID NITROGEN	NONPROTEIN NITROGEN (TOTAL AMINO ACID)
Rice starch (A)	210	177	33
Rice powder (B)	1030	845	185

The total amino acid content was estimated by the colorimetric method of Frame, Russel and Wilhelm (1943). The data from these determinations are recorded in Table 5.

Further analysis of the amino acid content was made to determine quantitatively the amounts of the following amino acids: arginine (McMahan and Snell, 1944; Shankman *et al.*, 1943b), aspartic acid (Stokes and Gunness, 1945), glutamic acid (Dunn *et al.*, 1944; Lyman *et al.*, 1945; Lewis and Olcott, 1945; Shankman *et al.*, 1943b), glycine (Dunn *et al.*, 1944; Shankman, 1943), histidine (Dunn *et al.*, 1945), isoleucine (Shankman *et al.*, 1943b), leucine (Shankman, 1943; Shankman *et al.*, 1943a; Schweigert *et al.*, 1944), lysine (Dunn *et al.*, 1944a), methionine (Shankman *et al.*, 1943a), phenylalanine (Dunn *et al.*, 1945a; Shankman *et al.*, 1943b), proline (Dunn *et al.*, 1944b), serine (Stokes and Gunness, 1945; Dunn *et al.*, 1944b), threonine (Shankman *et al.*, 1943a), tyrosine (Shank-

man *et al.*, 1943b), valine (McMahan and Snell, 1944; Kuiken *et al.*, 1943; Kuiken *et al.*, 1943a). Microbiological techniques were used for these determinations. Samples for both the total amino acid determination and individual amino acid assays were treated in the following manner: five hundred milligram amounts of the rice products were suspended in 8 ml. of 6 N hydrochloric acid in ampules. The ampules were sealed and then autoclaved at 115°C. and 15 pounds pressure for 6 hours. The contents of six ampules representing three grams of each of the test materials were pooled and filtered. The pH was adjusted to 6.8 with 4 N sodium hydroxide and the samples made up to a final volume of 100 ml. Aliquots of these preparations were used for all determinations. Corrections were made where needed for the additional salt present in the hydrolysate.

The results of the amino acid determinations and assays are found in Table 6.

TABLE 6

Approximate amino acid composition

Milligrams of amino acid per 100 grams of rice product

AMINO ACID	RICE STARCH (A)	RICE POWDER (B)
Arginine	173	430
Aspartic acid	76	580
Glutamic acid	136	1200
Glycine	92	122
Histidine	26	120
Isoleucine	430	2250
Leucine	188	520
Lysine	60	290
Methionine	34	270
Phenyl alanine	180	596
Proline	41	275
Serine	180	310
Threonine	93	428
Valine	32	224

Vitamins

I. *Water-soluble vitamins.* Assays were carried out for the following vitamins: Thiamine (Sarett *et al.*, 1944), riboflavin (Snell *et al.*, 1939), pantothenic acid (Skeggs *et al.*, 1944), pyridoxine (Atkin *et al.*, 1943), biotin (Wright *et al.*, 1944), p-aminobenzoic acid (Lewis, 1946), I-inositol (Atkin *et al.*, 1943), nicotinic acid (Association of Vitamin Chemists, 1947), folic acid (Landy *et al.*, 1941–42), choline (Glick, 1944), and ascorbic acid (Association of Vitamin Chemists, 1947a). With the exception of choline and ascorbic acid, all of the vitamins in this group were assayed by microbiological methods. Choline and ascorbic acid were determined chemically. Each assay was done in triplicate and the results were duplicated on samples prepared according to the assay procedure used for each vitamin. Results are tabulated in Table 7.

II. *Fat-soluble vitamins.* Assays were made only for carotene (Association of

Official Agricultural Chemists, 1945a) and Vitamin E (Emmerie *et al.*, 1939). Assays were not done for Vitamins A, D, and K. The absence of carotene precludes, in part, the presence of Vitamin A since cereal grains as well as most plant products are reported to be completely free of Vitamins A and D (Rosenberg, 1945). Rice flour has been used by Dam as a constituent of a diet to deplete chicks of Vitamin K (Dam, 1935). No assay was made for this vitamin since in Dr. Dam's opinion, expressed to us in a personal communication, many pounds of rice flour might contain only traces of Vitamin K, if any at all. Results are found in Table 7.

TABLE 7

Vitamins

Milligrams of vitamin per 100 grams of rice product

	RICE STARCH (A)	RICE POWDER (B)
Water soluble vitamins		
Thiamine	0.00	.038
Riboflavin	0.01	.046
Pantothenic acid	1.40	2.40
Pyridoxine	0.10	2.45
Biotin	.004	.006
P-aminobenzoic acid	0.00	.002
I-inositol	8.00	88.0
Nicotinic acid	0.40	3.55
Folic acid	0.00	0.00
Choline	45.0	100
Ascorbic acid	0.00	0.00
Fat soluble vitamins		
Vitamin A	—	—
Vitamin D	—	—
Vitamin E	2.45	3.25
Vitamin K	—	—
Carotene	0.00	0.00

DISCUSSION

The counts of amebae, shown in Table 1, were made under standard conditions, the only variable being the type of rice product used in the culture. The difference in yield can thus be attributed to a difference of constituents in the rice products.

The important role of proteins and their constituent amino acids in ameba culture is still being studied. It is of interest to note that the best growth was obtained with rice powder (B), the total nitrogen content of which is five times greater than the less nutritive rice starch.

All the amino acids assayed for have been shown to be present in rice powder (B) and these amino acids were present in relatively larger amounts in this product than in rice starch (A). The absence of tryptophane in all samples can be explained by its destruction during acid hydrolysis.

Some destruction of other amino acids also occurs during hydrolysis of complex molecules such as starch. Patten and Hill (1948) and Graham *et al.* (1949) have shown that amino acid concentrations are reduced by autoclaving in the presence of glucose. Despite these observations there is a significant difference between the nitrogenous constituents of rice powder (B) and rice starch (A). Further investigations into the role played by proteins and amino acids in the cultivation of *E. histolytica* is urgently needed.

Shafer, Ryden and Frye (1949) demonstrated that glucose appears to be an essential ingredient of media for ameba culture. We have likewise demonstrated the importance of carbohydrate (Hallman *et al.*, 1940). The figures in Table 3 show that there is nearly twice as much immediately water-soluble carbohydrate available from rice powder (B) as from rice starch (A) though the total carbohydrate is approximately the same.

The role of lipids in the culture of *E. histolytica* has been studied by Snyder and Meleney (1943). It is of interest that these workers found that an ether extract of rice powder could successfully replace cholesterol, the lipid under their consideration. Rice powder (B) contains five times as much lipid as rice starch (A). The relation of lipids to growth requirements still needs elucidation.

The role of vitamins in the culture of amebae has not yet been determined. Intestinal bacteria, which as a group most commonly accompany strains of *E. histolytica*, are able to synthesize many vitamins of the B complex, some of which may be essential to the culture of this ameba. Because rice powder and starches all show relatively large quantities of pantothenic acid, inositol, choline, and p-aminobenzoic acid, further investigation into their role in ameba culture is required.

Actually, *E. histolytica* may be grown without starches in many types of media, all of which are very complex. In simpler media however, addition of rice starch greatly facilitates growth. The amount of starch added to an ameba culture tube is small and the effectiveness of rice powder (B) in comparison with the rice starch (A), may be due to the fact that rice powder (B) in the amounts used provides amino acids, lipids, vitamins and carbohydrate in concentrations above the minimal critical levels required in any given culture.

As with any food substance, Bacto Rice Powder varies considerably from lot to lot. For example, nitrogen determinations on one lot gave a total nitrogen of 10.3 mg per gram, the lot used in this work, while the total nitrogen of a lot received at a later date contained 13 mg per gram. This variation could be attributed to differences in the origin of the raw rice and minor variations in its cultivation and manufacture. Every effort should be made to assure a standard composition of the materials used.

SUMMARY

A qualitative analysis has been made of a rice starch and a rice powder used in the cultivation of *E. histolytica*. The amount of total carbohydrate in each was determined as well as the content of quickly water-soluble carbohydrate. Rice powder (B) was found to contain a higher concentration of the quickly

water-soluble carbohydrate. Lipid determinations showed that rice powder (B) contains approximately seven times more total lipid and fatty acids than rice starch (A). Total nitrogen and individual amino acids were determined chemically and microbiologically. Rice powder (B) contains 5 to 6 times more nitrogen than does rice starch (A). Water soluble and fat soluble vitamins were determined. Rice powder (B) was found to be a richer source of these vitamins. Ash and moisture analyses were made on both products. Rice powder (B) was found to possess a higher inorganic content than rice starch (A).

It is believed that this information may assist in determining which nutritive factors present in rice powder (B) may be required in a completely synthetic medium.

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FURTHER STUDIES ON THE CULTIVATION OF THE AVIAN MALARIA PARASITES[1]

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It is certain that anything like complete understanding of the physiology of the malaria parasites, and perhaps also of the determining factors in their life cycle, will not be attained until it is possible to cultivate all the species on artificial media of known composition. This is true even though rather intensive studies of several of the more pathogenic human and simian species of plasmodia have already been made. From a purely biological point of view an understanding of the physiological factors determining host-parasite relationships is equally important for all the species of plasmodia. When we are able to cultivate the malaria parasites under conditions which really approach those existing in the natural host, we may also expect to learn much of plasmodial genetics and perhaps to draw plausible inferences at least as to how these parasites evolved. Indeed the culture tube may be regarded as a kind of artificial host with the great advantage of having fewer variables.

For this reason we have been interested for some time in attempts to cultivate the avian plasmodia, a group comprising almost a third of the known species of the genus. The relative success of the culture method devised by Ball, Geiman, and their associates (Ball *et al.*, 1945; Geiman *et al.*, 1946) for simian and human malaria parasites made us anxious to apply it to the closely related forms of birds. The results of earlier experiments have already been reported (Manwell and Brody, 1950). Although none of the four species used in that work, *Plasmodium cathemerium*, *P. relictum*, *P. gallinaceum* and *P. lophurae*, showed an *in vitro* growth and multiplication comparable to that of the mammalian plasmodia under similar conditions, the matinal variety of *P. relictum* did fairly well, and it seemed desirable to continue and extend such studies.

The experiments now being reported were done mainly with *P. cathemerium*, one of the four species previously used, and with *P. hexamerium*. Work with the latter seemed especially important because it belongs to a group of avian plasmodia the members of which differ from most others in their relatively small size, in the small number of merozoites produced per segmenter, and in the chronic nature of the infection. All are characterized by elongate gametocytes. Other species in the group, which thus appears to be a natural one, are *P. vaughani*, *P. nucleophilum*, *P. rouxi*, and very probably *P. polare*. So far no other work on the cultivation of any of these species has been reported to our knowledge. Several experiments were also done with *P. relictum*, using the same strain as that employed in previous studies, with the idea of confirming earlier results and for the purpose of comparison. Subculturing, not tried before, was also attempted.

[1] This work was aided in part by a grant from the National Institutes of Health.

MATERIALS AND METHODS

The strain of *P. cathemerium* was isolated from a red-winged blackbird, *Agelaius phoeniceus phoeniceus*, caught in Fayetteville, N. Y. (a village about 8 miles east of the University) on May 25, 1950, and has been designated "3F" by the Committee on Terminology of Strains of Avian Malaria of the American Society of Parasitologists. The strain of *P. hexamerium* was originally secured from a Lincoln's sparrow, *Melospiza lincolni lincolni*, caught in the same place on May 15, 1947. It has been designated the "10D" strain. Both strains have been maintained in canaries except as they have been required for cultivation or chemotherapeutic experiments. For these purposes White Pekin ducks have been used. Ducklings are very susceptible to both strains, although it required considerable time and a good many attempts before *P. hexamerium* would produce consistently heavy infections. The characteristics of this species in the duck have been described elsewhere (Manwell, 1952).

The ducklings were secured from a hatchery in Ohio immediately after hatching, and were generally inoculated within the first week. Older ducks, though they retain malaria infections more or less indefinitely, are more refractory to inoculation. Blood for experimental purposes was withdrawn from the jugular vein in most cases, after first removing the down, and new infections were produced by inoculation of parasites via the same route.

In all the experiments the Harvard medium in unmodified form was used in rocker perfusion tubes, and the methods were in general the same as in the work previously reported (Manwell and Brody, 1950). However subcultures as well as primary cultures were also attempted, generally after an initial culture period of 24 hours. For this purpose, after initial microscopic examination to determine the parasite population attained, the cultured blood was removed from the culture tube with a sterile pipette, mixed with an equal quantity of fresh blood from a very young duckling (such blood is especially rich in reticulocytes, which seem particularly receptive to merozoite invasion in the case of *P. cathemerium*, though not of *P. hexamerium*), again checked microscopically for parasite concentration and placed in another culture tube with a fresh supply of medium.

Three methods were used to evaluate the success of the cultures: (1) parasite counts from J.S.B.-stained smears to show the ratio of the final to the original count per unit number of red cells, constituting what we have called the "Growth Index"; (2) the staining characteristics of the cultured parasites as compared with those in fresh blood; and (3) viability as indicated by the length of the incubation (prepatent) period and character of the infection in ducklings and canaries inoculated from the cultures. Infections exhibiting short incubation periods and relatively high virulence following inoculation of parasite-containing blood are usually the result of a high parasite dosage, which in this case would mean relatively successful culture.

RESULTS

P. hexamerium gave fairly good growth. The mean 24 hour Growth Index was about 1.3 on primary cultures, and even better (about 1.8) on subcultures (see

Table 1), although this latter figure may not be significant, since there is a high degree of variability in the results of experiments of this kind. In evaluating these results it must be remembered that the usual number of merozoites per segmenter in this species is only six, which necessarily means a slower rate of increase[2] and a lower Growth Index than for species such as *P. cathemerium* or *P. relictum*, in

TABLE 1

Cultivation experiments with Plasmodium hexamerium (*"10D" strain*)

A. Primary cultures (7 experiments)

EXPERIMENT NO.	NUMBER OF TUBES	GROWTH INDEX			VIABILITY (72 HRS.) AND INCUB. PERIOD
		24 hr.	48 hr.	72 hr.	
					days
18	4	1.35	1.29	0.44	5.8
19	1	1.59	1.06	—	7.0
20	4	0.89	0.89	0.90	6.0
21	2	1.41	—	—	*
22	4	1.18	1.63	—	*
26	5	1.38	1.17	1.10	†
27	5	1.42	1.41	1.41	5.1
Total.....	25				
Means.....		1.31 ± 0.03	1.24 ± 0.01	0.96 ± 0.24	5.9

B. Subcultures (2 experiments)

EXPERIMENT NO.	NUMBER OF TUBES	SUBCULTURED FROM EXP. NO. (AFTER 24 HRS.)	GROWTH INDEX		VIABILITY (48 HRS.) AND INCUB. PERIOD
			24 hrs.	48 hrs.	
					days
24	2	21	1.63	—	3.9
25	2	22	1.97	1.27	4.0
Total.....	4				
Means.....			1.80	1.25	3.95

* Viability tests carried out on subcultures.

† Viability tests not carried out, due to temporary lack of birds.

which the corresponding merozoite figure is more than double that of *P. hexamerium*.

Forty-eight hour cultures also showed a fairly high mean Growth Index (1.2), and even in the 72 hour cultures there was a high rate of survival as evidenced by a Growth Index of almost 1.0. Viability tests showed that the parasites also retained their ability to produce good infections, as evidenced by short incubation periods and a normally high parasitemia in subinoculated birds. Ducklings inoculated from 24-hour cultures developed patent infections after an incubation pe-

[2] The length of the asexual cycle is approximately 24 hours, but it is much less definite than with species such as *P. cathemerium*, and *P. relictum* var. *matutinum*.

riod averaging only 3.9 days, and after 48 hours of cultivation it was scarcely longer (4.0 days). Seventy-two-hour cultures gave an incubation period averaging 5.9 days. These figures are to be compared with those obtained when blood

TABLE 2

Cultivation experiments with Plasmodium cathemerium (*"3F" strain*)

A. Primary cultures (9 experiments)

EXPERIMENT NO.	NUMBER OF TUBES	GROWTH INDEX*		VIABILITY (AFTER 48 HRS.) AND INCUB. PERIOD
		24 hrs.	48 hrs.	
				days
8	2	1.08	1.54	6
10	5	1.78	1.44	6
11	4	1.18	1.06	§
12	3	0.92	†	‖
13	2	1.31	†	‖
14	2	1.13	0.90	5
15	3	0.90	†	‖
16	2	1.07	†	‖
17	3	0.85	†	‖
Total	26			
Means		1.13 ± 0.01	1.24 ± 0.14‡	5.7

B. Subcultures (5 experiments)

EXPERIMENT NO.	NUMBER OF TUBES	SUBCULTURED FROM EXP. NO.	GROWTH INDEX 24 HRS.	VIABILITY (48 HRS.) AND INCUB. PERIOD
				days
28	2	12	1.54	7.0
29	2	13	1.06	§
30	2	15	0.95	8.7
31	3	16	1.08	5.5
32	2	17	0.94	§
Total	11			
Means			1.11 ± 0.11	7.0

* Both the 24 hour and the 48 hour Growth Index were calculated with reference to the *initial* degree of parasitemia in the cultures.

† Subcultured at the end of the first 24 hours.

‡ Mean calculated only from experiments not subcultured.

§ Viability tests not done due to temporary lack of birds.

‖ Viability tests carried out on subcultures.

from an active case of *hexamerium* malaria is inoculated, and although the incubation period in such cases varies considerably according to the number of parasites introduced, in our work it generally approximated 4 days.

We used *P. cathemerium* in this study largely because previous work on it

(Manwell and Brody, 1950) had given especially poor results and we wished to see whether we could better them. We also desired to compare it with *P. hexamerium*, a species which differs from it in many important ways. The results for *P. cathemerium* are summarized in Table 2.

Although a somewhat higher Growth Index (about 1.3 for 24 hour cultures) was obtained than in the earlier work just referred to, results were still disappointing. For 48-hour cultures, the Index was about 1.2, and 24-hour subcultures gave a corresponding figure of only 1.1. When the high rate of multiplication of this

TABLE 3

Cultivation experiments with Plasmodium relictum

A. Primary cultures (5 experiments)

EXPERIMENT NO.	NUMBER OF TUBES	GROWTH INDEX		VIABILITY (AFTER 65 HRS.) AND INCUB. PERIOD
		24 hr.	48 hr.	
				days
1	6	1.39	—	*
2	2	0.86	0.67	nonviable
3	2	1.45	0.60	4.8
6	2	2.55	—	*
9	3	1.06	—	*
Total	15			
Means		1.41 ± 0.14	0.62	4.8

B. Subcultures (2 experiments)*

EXPERIMENT NO.	NUMBER OF TUBES	SUBCULTURED FROM EXPERIMENT NO.	GROWTH INDEX 24 HRS.
6a	2	6	1.00
9a	3	9	1.06
Total	5		
Means			1.00 ± 0.14

* Viability tests not done due to temporary lack of birds.

species under natural conditions is considered, it is apparent that this culture medium and technique are very poorly adapted to its requirements. Viability tests supported this interpretation. Although subinoculated birds developed infections from both primary and subcultures, the incubation period was abnormally long (6 or 7 days, as compared with a normal figure of 3 or 4). Thus it appears not only that there was little multiplication in the cultures, but that the parasites were probably somewhat impaired physiologically, so that their degree of infectiousness was lessened. Abnormal staining of many of the parasites suggests the same conclusion.

The work done with *P. relictum* was limited in amount and gave somewhat lower rates of multiplication than those previously reported (Manwell and

Brody, 1950). It is summarized in Table 3. For 24-hour cultures a Growth Index of about 1.4 was obtained—considerably less than the corresponding figure of 2.12 previously secured. In one experiment, however, a Growth Index of 2.55 was gained. Parasites were found viable after a maximum cultivation period of 65 hours. Subcultures, not attempted in the previous work, gave a mean 24-hour Growth Index of 1.0, which indicates only survival, although two tubes gave indices slightly higher (about 1.2) suggesting some multiplication.

DISCUSSION

These results confirm the evidence of previous studies that the Harvard medium is not well adapted to the physiological requirements of the avian plasmodia, but they also show that these species differ considerably among themselves in this regard, and they emphasize the need for further experiments of this sort.

Although multiplication occurs in some of the tubes at least during the first 24 hours of culture of every species we have tried, there is much variation even in the case of a given species. The results of the Harvard group on the mammalian plasmodia were also highly variable, even though growth rates in their cultures consistently exceeded ours.

Subcultures also showed a good deal of variability, and (except in the case of *P. hexamerium*), gave lower rates of multiplication. It is thus evident that much remains to be done before a really useful method of cultivation of the malaria plasmodia is developed. Such a medium and process should give multiplication at least equal to that occurring in the natural host and indeed it should better it, for the factor of natural resistance due to the defensive mechanism of the host will be absent. A certain proportion of the parasites in the culture should become gametocytes. This is a factor which has so far been largely ignored in the evaluation of successful cultivation. The results of cultures done under similar conditions should be consistent, whereas at present there is at best a high degree of variability. It should, of course, also be possible to make subcultures almost indefinitely.

It is certainly true that much progress has been made since the early experiments of Bass and Johns, and of the many who confirmed and tried to improve on the work of these pioneers. Perhaps the greatest advance is the development of a medium of synthetic and known composition (although even now some blood must be used, for we cannot yet duplicate with any exactness the normal intracellular environment of the parasite). As a result, we now know much about the basal physiology of the malaria plasmodia (cf. McKee *et al.*, 1946; Moulder, 1948). But we are still far from the degree of success attained in cultivating such other parasitic protozoa as the intestinal amebae, the trypanosomes and the leishmanias.

SUMMARY

Experiments involving the cultivation of three species of avian plasmodia, *Plasmodium cathemerium*, *P. hexamerium* and *P. relictum*, on the Harvard me-

dium show the medium to be best adapted to the requirements of *P. hexamerium*. Although the Growth Index (defined as the ratio of parasites per unit number of red cells at the end of a given period of cultivation to that at the start) was numerically not as high as that obtained with some other species of avian plasmodia, it represents a relatively better result because this species has a much slower rate of natural increase. Viability tests and staining reactions of cultured parasites confirm this conclusion. Relatively good results were also obtained in subculture. Nevertheless even this species does not show a rate of multiplication in culture approaching that observed *in vivo*.

Of the other two species, *P. relictum* (as previously reported) does moderately well in primary cultures, but little more than survival occurs in subcultures. *P. cathemerium* shows even less multiplication in primary cultures, and virtually none in subculture.

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CONTINUOUS PROPAGATION OF *PLASMODIUM GALLINACEUM* IN CHICKEN ERYTHROCYTES

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This article is a report of some experiments that have been performed to determine the conditions under which continuous growth and multiplication of the erythrocytic stage of *Plasmodium gallinaceum* can be maintained *in vitro*. *P. gallinaceum* was selected, not because of any special characteristics it might have had for cultivation, but rather because it had been deemed most suitable for the type of approach to the general problem of malaria chemotherapy which is being used in this laboratory.

Previously, the most successful work on the cultivation of the erythrocytic phase of malaria parasites was that described by Ball and his colleagues (1945) with *Plasmodium knowlesi* and that of Trager (1947) with *Plasmodium lophurae*. The former investigators observed multiplication throughout the course of a week, but their data indicated that the rate of multiplication steadily decreased after the first few subcultures. Trager reported active multiplication throughout a series of three successive subcultures; during the eight days of these experiments the parasites increased 170-fold. In both cases the cultures consisted of a mixture composed of various nutritional substances and salts, together with some normal homologous serum. The nutritional substances were mainly those found to be metabolized by parasitized cells or considered to be of likely nutritional value to them. The cultures were incubated in a suitable atmosphere of carbon dioxide and air, and the cells were maintained in suspension by continuous rocking of the culture.

The present study was begun by trying to cultivate *P. gallinaceum* under the conditions described by the above-mentioned workers. The results showed that the rate of development of the parasites was considerably less than the normal rate and that their appearance in stained smears was not good. (Manwell and Brody, 1950, have already published an article in which they reported that this type of culture was not favorable for *P. gallinaceum*.) Efforts were then turned to a study of the growth and multiplication of *P. gallinaceum* in a medium composed chiefly of blood, on the theory that such a system would be most likely to contain the essential nutrients for the cells and parasites and would consequently facilitate determination of the other factors important to cultivation.

MATERIALS AND METHODS

Source of parasites. The strain of *P. gallinaceum* employed was originally obtained by this laboratory from Beltran in Mexico in 1941. It has been maintained since that time in chickens by blood and mosquito transfer. In most of the experiments the parasitized blood for seeding the cultures was obtained from 1- to 2-week-old infected chicks. The details of infecting the chicks have been re-

ported by Whitman (1948).[1] Briefly, the procedure consisted of inoculating intravenously a suspension of sporozoites prepared from the salivary glands of infected *Aedes aegypti* mosquitoes into 1- to 7-day-old chicks. Seven days afterward the blood contained malaria parasites almost all of which were in the early trophozoite stage. The dosage of sporozoites was adjusted to try to produce a parasitization of between 10 and 30 per cent of the red cells on the seventh day. A group of six chicks was usually inoculated to ensure that there would be at least one satisfactory for an experiment. Only chicks having a very phasic infection were used to provide parasites for seeding the cultures. It has been estimated that the majority of parasites were from 4 to 8 hours old when added to the cultures, with the remainder being from a few minutes to perhaps 12 hours old. During the early part of the work, White Leghorn chicks were used, but later White Rock chicks were substituted and no significant difference was noted in the results.

Apparatus. The apparatus employed in these experiments was similar to that described by Geiman *et al.* (1946). The rocker moved through an arc of about 90 degrees during half a cycle. A rocking rate of about 13 cycles per minute was found adequate for optimum growth and multiplication of the parasites. The culture vessels were ordinary wide-mouthed jars of approximately 60 ml. capacity, fitted with rubber stoppers containing inlet and outlet tubes for the continuous passage of a gas mixture during incubation.

Preparation of the cultures. In preparing a culture, the infected chick blood was diluted with normal chicken blood. The normal blood was obtained from adult White Leghorn or White Rock chickens. Heparin was added to the blood in the ratio of one part of a 200 mg. per cent heparin solution in saline to nine parts of blood. The total volume of each culture was from 5 to 8 ml. The endeavor was to start the cultures with approximately one to two per cent of the erythrocytes parasitized.

From 0.15 to 0.25 ml. of a 5 per cent solution of glucose was added to each culture. Also, sufficient penicillin (Penicillin G-K salt) was added to produce a final concentration in the culture of about 400 units per milliliter. The pH of the culture was satisfactorily maintained by passing 7 per cent carbon dioxide—93 per cent air mixture continuously through the vessels during incubation. Precautions were taken during the handling of the cultures to avoid any undue rise in the pH. The temperature range from 40°C. to 42°C. was found optimum for parasite growth.

Technique of subculture and change of medium. Subcultures were made by simply diluting the culture in freshly prepared equilibrated erythrocyte suspensions or whole blood, as the experiment required. In the short-term experiments, subcultures were made after 20 to 24 hours incubation. Usually a 1:5 dilution of the original culture was made.

In changing the medium it was found that centrifugation at 1,200 r.p.m. for 10 to 15 minutes effectively sedimented the cells without detectable damage.

[1] All of the sporozoite suspensions used for infecting the chicks were obtained through the courtesy of Dr. Loring Whitman.

Preparation of dialyzed chicken erythrocyte extract (CEE)-plasma dialysate medium. The preparation of the erythrocyte extracts was essentially the same as that described by Whitman (1948). To prepare the plasma dialysate, normal chicken plasma containing heparin was dialyzed against several changes of distilled water. The total dialysate was pooled and desiccated from the frozen state *in vacuo*. Following desiccation the residue was dissolved in a volume of distilled water equal to one-half the original volume of plasma. The final medium was composed of equal parts of the final dialysate solution and the dialyzed CEE. The medium was equilibrated to the carbon dioxide-air mixture before use. Both the plasma dialysate and the dialyzed CEE were prepared from originally sterile material. However, the final product was not considered sterile. No bacterial growth was observed in any of the cultures employing this medium.

Preparation of heated serum-erythrocyte lysate medium. The medium was prepared from normal chicken blood defibrinated by shaking with glass beads. The blood was transferred to sterile 20 ml. lusteroid tubes and centrifuged in order to pack the cells. The serum was carefully separated and heated for an hour at 60°C., following which it was cooled and then equilibrated to 7 per cent carbon dioxide—93 per cent air mixture. Meanwhile, the lusteroid tube containing the packed cells was placed in a thermos containing dry ice. After thoroughly freezing and shortly before the heated serum was equilibrated, the packed cells were removed and allowed to thaw slowly at room temperature. The equilibrated heated serum was then added to the lysed cells, and the whole was thoroughly mixed with a pipette. The suspension was centrifuged for an hour in the cold in a high-speed angle centrifuge at about 10,000 r.p.m. The supernatant liquid was carefully removed and transferred to pyrex ampoules, shell frozen in a dry ice and alcohol bath, and stored in the dry ice chest. Prior to use, the material was thawed and again equilibrated to 7 per cent carbon dioxide—93 per cent air mixture. A sample of each batch was cultured in broth at the time the final product was ampouled.

Determination of growth and multiplication. The availability of parasitized cells in phase in the early trophozoite stage for seeding the cultures made it very convenient to follow the course of development of the parasites during a single cycle. Routinely, smears were made at the beginning, after the first day, and at the end of an experiment on the second day; additional smears were made as needed. A differential parasite count of the initial smears and the smears made after the first day's cultivation served as an index of growth. Ordinarily, 50 consecutive parasites were classified as trophozoites, schizonts, segmenters, gametocytes, or unknown, and the results were expressed as the percentages of the various forms. Parasite counts of the initial and final smears determined the extent of multiplication that had occurred. The results of the parasite counts were expressed as the numbers of parasites per 10,000 r.b.c.

All final smears were stained by the Giemsa method, slightly modified from the technique of Wilcox and Logan (1941). Whenever a quick stain was desired, Seller's technique was used. Later, the rapid Giemsa method described by Singh and Bhattacharji (1944) was found satisfactory for most work. Seller's method

had the disadvantage of not staining the nuclei of the parasites sufficiently to enable an accurate determination of the stage of the parasite. The Singh technique was found difficult to use in the presence of large amounts of extracellular hemoglobin.

EXPERIMENTAL

Before describing the technique which permitted continuous subculture of the parasites, it is desirable to present briefly the results of some experiments related to the general problem.

The influence of the oxygen tension on the growth of parasites. Anfinsen *et al.* (1946) reported that *P. knowlesi* grew as well under very reduced oxygen tension as it did when the tension was about that of air. When the erythrocytic stage of *P. gallinaceum* was cultivated in an atmosphere of 5 per cent carbon dioxide—95 per cent nitrogen, very little, if any, growth could be detected.

TABLE 1

Influence of erythrocyte concentration on the in vitro *growth of* P. gallinaceum

CONCENTRATION OF RED CELLS	HOURS OF CULTIVATION	RATE OF ROCKING CYCLES/MINUTE	DIFFERENTIAL PARASITE COUNT				
			Per cent				
			Trophozoites	Schizonts	Segmenters	Gametocytes	Unknown
Whole blood	24	6	26	48	4	22	0
½ Whole blood	24	6	42	44	0	12	2
Whole blood	23½	17	14	68	2	12	2
½ Whole blood	23½	17	50	36	0	2	12

The growth of *P. gallinaceum* was markedly inhibited in the presence of a high oxygen tension. A similar result has been reported for other malaria parasites by Trager, Anfinsen, and others.

The influence of erythrocyte concentration on parasite growth. The effect of red cell concentration on the growth of parasites was observed early in the work and a great deal of effort has since been devoted to trying to understand the phenomenon. The influence of erythrocytes was clearly demonstrated by comparing the first 24 hours' growth obtained in cultures containing the same concentration of red cells as whole chicken blood with the growth in cultures containing half that concentration. In these experiments the whole chicken blood was seeded with parasitized cells, and from this the dilute cell suspensions were prepared by adding equal volumes of plasma. In this way the ratio of parasites to red cells in a given experiment remained constant. The results of two such experiments have been summarized in Table 1. The data show that a decrease in the erythrocyte concentration produced a generally inhibitory effect on the growth rate of the parasites, as manifested by the smaller proportion of parasites that developed into gametocytes and segmenters. It was also seen that the schizonts in the

cultures with the lower concentrations of erythrocytes were smaller in size and contained fewer nuclei. The cultures with still lower concentrations of red cells grew so poorly that the degenerative changes present in many of the parasites rendered their identification difficult.

Growth and multiplication of parasites in dialyzed chicken erythrocyte extract (CEE)-plasma dialysate medium. Normal chicken erythrocytes were suspended in this medium to produce a red cell concentration equivalent to that of whole chicken blood. The normal cell suspension was then seeded with a volume of infected blood not exceeding 0.3 ml. per culture, which was not considered to add a significant amount of plasma. (In some later experiments with this medium, results were obtained which suggested that the small amount of plasma present in the earlier experiments had a stimulative effect on the parasites.) After about 24 hours' incubation, subculture was made to freshly prepared normal cell suspensions. Control cultures were prepared in whole chicken blood.

TABLE 2

Comparison of in vitro *growth of* P. gallinaceum *in whole blood and in dialyzed CEE—plasma dialysate medium*

EXPERIMENT NO.	MEDIUM	DIFFERENTIAL PARASITE COUNT AFTER 24-HOUR CULTIVATION				FOLD INCREASE AFTER 46 HRS.
		Per cent				
		Tropho-zoites	Schi-zonts	Seg-menters	Gameto-cytes	
1	Control	36	44	4	16	4.2
	10% CEE—plasma dialysate	56	40	0	4	5.4
	25% CEE—plasma dialysate	54	42	2	2	3.3
2	Control	30	48	2	10	1.1
	25% CEE—plasma dialysate	60	38	0	2	4.5

Typical results obtained with this medium have been summarized in Table 2, together with the results obtained in the control cultures. The differential counts made on smears prepared after a day's cultivation indicate that the rate of development of the parasites in the CEE-plasma dialysate medium was slower than that of parasites in the control cultures. Although the growth rate was retarded, the parasites had a very healthy appearance in the stained smears. It was noted also that the majority of segmenters which developed in the CEE-plasma dialysate medium contained fewer merozoites (8–12) than did those formed in the control cultures. In spite of the delay in the rate of development, the parasites in the CEE-plasma dialysate multiplied at a rate equal to and often greater than that in the whole blood medium. The retardation in growth rate observed in the CEE-plasma dialysate medium was similar in extent to that observed in the cultures which contained a concentration of erythrocytes half that of normal whole chicken blood. However, the differences in appearance of the parasites in stained films and the differences in multiplication rate between the two preparations suggest that the mechanisms underlying the decrease in growth rate in each instance were different.

The effect of heated plasma on the growth and multiplication of parasites. Exposure of plasma and serum to temperatures of 56°C. to 65°C. has been found by many workers to remove various kinds of inhibitory activity. Since some evidence pointed to the possibility that an inhibitory factor was active in many of the cultures, it was considered worthwhile to determine the influence of heated plasma on the growth of parasites. Whole blood cultures were prepared in pairs which were duplicates in all respects except that the plasma of one had been previously heated to a temperature of 56°C. for an hour and the plasma of the other had not. (The heat caused the formation of a certain amount of precipitate which was removed by centrifugation.) Before mixing with the cells, the heated plasma was equilibrated with the carbon dioxide and air mixture to restore the hydrogen ion concentration to normal. Differential parasite counts made on smears prepared from each type of culture after a day's growth were not significantly different. However, it was noted at that time that the parasites present in the cultures which contained heated plasma had a healthier appearance in stained smears than those in the control cultures. The multiplication rate in the cultures with the heated plasma was greater than that in the cultures which contained unheated plasma. What was considered more important, however, was the observation that the young trophozoites present at the end of the experiment had grown more in the cultures with the heated plasma than in the control cultures with unheated plasma.

The effect of calcium chloride on parasite multiplication. When 0.3 ml. of a 0.1 molar solution of calcium chloride was added to the usual whole blood culture, the parasites grew as well as in the control cultures, but multiplication was almost completely suppressed. Gametocytes, however, were present in numbers comparable to those in the control cultures. Less marked and more variable suppression of multiplication was obtained when isotonic solutions of magnesium or potassium chloride were added to the cultures.

Subculture experiments. Under proper conditions whole blood cultures produced a regular but variable increase in the parasite count during the first cycle. Attempts to subculture this type of preparation, however, uniformly failed.

As noted previously, one of the chief differences between the cultures with heated plasma and those with unheated plasma was that in the former the young trophozoites present at the end of the experiment had grown more. Only a single subculture experiment was done with the heated plasma type of culture. Six subcultures in all were made, and in each, by means of frequent smears, a succession of healthy appearing parasites in various stages of development was observed in spite of the fact that multiplication was not sufficient to prevent a constant fall in the parasite count. At the end of the sixth subculture, a group of chicks was inoculated intravenously with samples of the subculture and all became blood positive within a few days.

At one time during this study chicken erythrocyte extracts, prepared by the method described by Whitman, were added to the cultures in small amounts. No significant effect was observed on the rate of growth and multiplication of the parasites and this approach was abandoned. The technique of preparing a

concentrated erythrocyte lysate, devised by Trager (1950), suggested the possibility of adding large amounts of erythrocyte material to the cultures. In a preliminary experiment in which an erythrocyte lysate preparation was incorporated into a heated plasma type of culture, better growth was obtained in the first subculture than had been obtained in any previous experiments. A subculture series was begun and carried through eight consecutive transfers, at which time it had to be discontinued because of contamination of the culture. The information obtained indicated that the multiplication was sufficient to overcome the dilution factor and maintain a relatively constant parasite density. This experiment was repeated and is described in detail in the following paragraphs. After ten successive subcultures, the experiment was terminated as the objective was considered to have been accomplished.

TABLE 3

Continuous subculture of P. gallinaceum *in heated plasma-erythrocyte lysate medium*

SUBCULTURE NO.	TIME OF SUBCULTURE IN HOURS	DILUTION AT TIME OF SUBCULTURE	ACCUMULATED DILUTION OF SUBCULTURES	PARASITE COUNT PER 10,000 R.B.C.	DIFFERENTIAL PARASITE COUNT			
					Per cent			
					Tropho-zoites	Schi-zonts	Seg-menters	Gameto-cytes
0	0			129	98	1		1
1	24	1:5	1:5	95	26	52	4	18
2	65	1:3	1:15	99	53	37	5	5
3	104	1:3	1:45	37	44	48	8	0
4	145	1:3	1:135	35	72	24	4	0
5	186	1:2	1:270	—	—	—	—	—
6	227	1:2	1:540	35	94	0	3	3
7	266	1:3	1:1,620	59	94	6	0	0
8	306	1:3	1:4,860	61	100	0	0	0
9	346	1:2	1:9,720	28	93	7	0	0
10	386	1:2	1:19,440	41	87	10	3	0
—	427	—	—	88	82	18	0	0

In this subculture series, normal erythrocytes, in a concentration equivalent to that of whole normal chicken blood, were suspended in heated serum-erythrocyte lysate medium and seeded with infected chick blood. To each culture was added 0.15 ml. of 5 per cent glucose solution and 2,000 units of penicillin contained in a volume of 0.1 ml. of saline solution. The total volume of each culture was from 5 ml. to 7 ml., the variation being due to the convenience of preparing the different dilutions.

The essential data obtained from this experiment have been summarized in Table 3. The information as to the times at which the subcultures were made is expressed as the number of hours that had elapsed from the beginning of the experiment. The dilution made at the time of each subculture is included, together with the total dilution of the original culture that had accumulated at the time. Actually, the figures represent minimum dilutions because no effort was made to compensate for the cells lost during the manipulations. The medium in the culture

was changed at least once every 24 hours. Smears were generally prepared at the time the subculture was made.

The results indicate that although the count fluctuated somewhat during the period of cultivation, it was possible to maintain a relatively constant parasite density. As yet, no study has been made of the factors that might contribute to such a fluctuation. It has been considered that the time at which the subcultures were made might be of importance. When the experiment was begun, it was hoped that a good proportion of the parasites would tend to develop as a group and that the subculture interval could be determined by following parasite growth by means of frequent smears. However, the phasic nature of the original starting material disappeared after the first subculture. Reliance was then placed on the finding of well developed schizonts or segmenters at a reasonable time after the previous subculture. Toward the end of the series it was felt that a certain periodicity was again established, as the parasites were more uniform and looked healthier as a group than at any time after the first subculture.

Samples from the first, sixth, and final subcultures were inoculated intravenously into groups of three chicks. The same volume was administered to each chick. The chicks were from two to four days of age at the time of inoculation. The response of the chicks was determined from daily smears and from the average day of death. On this basis there was no significant difference among the three groups, which meant that the inocula contained essentially the same numbers of viable parasites. Two of the chicks inoculated with samples from the final subculture were fed upon by *Aedes aegypti* shortly before their deaths. Exflagellation was observed in the usual stomach preparations of the mosquitoes that fed on the chicks. Several days later typical oocysts were found in the stomachs of these two groups of mosquitoes. Sixteen days after the infective feeding, mosquitoes from each group were fed on separate chicks and transmission was obtained in each instance.

SUMMARY

In vitro studies of the growth of *P. gallinaceum* in normal chicken plasma showed that an erythrocyte concentration equivalent to that of normal blood was most favorable to the parasite. Exposure of the plasma portion of a culture to 56°C. for an hour prior to mixing with the erythrocyte was found to increase parasite growth and multiplication. This effect was thought to be due to the heat lability of some inhibitory system present in normal chicken plasma.

Continuous growth and multiplication of *P. gallinaceum* was obtained in erythrocytes suspended in a medium composed of heated plasma and erythrocyte lysate. During a series of ten successive subcultures the parasite density was maintained at a relatively constant level, while the dilution factor of the original culture rose to 1:19,440. The parasites of the final subculture reacted in young chicks and in *A. aegypti* mosquitoes in a normal manner.

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CHEMOTHERAPY OF EXPERIMENTAL CHAGAS' DISEASE WITH THIRTY ANTIBIOTICS[1]

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Although numerous drugs and a few antibiotics have been tested thus far against *Trypanosoma cruzi* infections in man and in experimental animals, no specific drug has yet been found which will produce an absolute cure for Chagas' disease.

The antibiotics which have been tested up to the present against *Trypanosoma cruzi in vitro, in vivo,* or in both, consist of the following: aureomycin, chloramphenicol, gramicidin, penicillin G sodium, streptomycin, terramycin, and tyrocidine. Of these, only penicillin has been used thus far in clinical treatment of human cases of Chagas' disease. Penicillin G sodium was reported first by Mazza (1945, 1950) to show pronounced clinical action in doses of 20,000 to 30,000 Oxford units per kilogram of body weight, when 10,000 to 20,000 units were given intramuscularly every three hours. He states, however, that its trypanocidal effect was low. Talice (1945), in treating one acute case of Chagas' disease in an 11-year old boy, concluded that the treatment with this antibiotic had no beneficial action either from clinical or parasitologic standpoints. Earle (1946) and Laranja, Dias and Nobrega (1948) have likewise reported discouraging results with penicillin in treating human cases of Chagas' disease.

Neghme (1945), in treating experimental Chagas' disease in mice with penicillin, concluded that this antibiotic had no effect in suppressing or curing the disease. Jarpa *et al.* (1949, 1950) found that the administration of aureomycin to mice experimentally infected with *Tr. cruzi* resulted in an increased severity of the disease, and the addition of aureomycin to cultures of *Tr. cruzi* had no apparent effect upon the multiplication or viability of the parasite. The same investigators also report unsuccessful results with streptomycin and chloramphenicol in mice inoculated with a Chilean strain of *Tr. cruzi*. Action of these antibiotics upon cultures of the trypanosome was likewise ineffective. Agosín *et al.* (1950) found that terramycin was without value in altering the course of infection with two Chilean strains of *Tr. cruzi* in mice, and the antibiotic had no effect on cultures of the trypanosome *in vitro*. Weinman (1947) found that tyrocidine *in vitro* produced changes in *Tr. cruzi* cultures. This trypanocidal property of tyrocidine was confirmed by Amrein (1950). Bettinotti (1951) tested aureomycin, chloramphenicol, penicillin, streptomycin and terramycin alone and in various combinations against *Tr. cruzi in vitro*. He found none of these six anti-

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biotics had any trypanocidal effect on *Tr. cruzi* when used alone; however, he believed "a synergistic rather than an additive action" was obtained when chloramphenicol and terramycin were used together.

The object of the present communication is to report the results obtained with thirty antibiotics against *Tr. cruzi* both *in vivo* and *in vitro*. Twenty-three of these antibiotics are tested on *Tr. cruzi* both *in vivo* and *in vitro* for the first time.

MATERIAL AND METHODS

Strains of Trypanosoma cruzi. Two strains of *Trypanosoma cruzi* were used in this study. The first strain, designated as Pack-Brazil-46, was isolated directly from a human patient in Brazil by Packchanian during 1946. The subcultures of this strain were made monthly or bimonthly on blood agar slants containing about 20 to 30 per cent defibrinated rabbit blood. This strain, when inoculated into mice (*Mus musculus*) directly from blood agar slants, produces a chronic infection.

The second strain, WBH (Wellcome Brazil Hamburg) was received through the courtesy of Dr. T. S. Hauschka of Philadelphia, Pa., during 1950. On arrival in Texas, it was cultured on Novy and MacNeal's medium, and, after passage through mice and *Triatoma gerstaeckeri*, was re-exalted to its original virulence to mice. This strain is designated as WBHT (Wellcome Brazil Hamburg Texas) in order to distinguish it from the original strain described by Hauschka (1950). The *Tr. cruzi* WBHT strain was maintained in mice (*Mus musculus*) by weekly transfer of heart blood from infected to normal mice. When infected blood containing at least 500,000 metacyclic trypanosomes per inoculum was injected intraperitoneally into normal mice, acute and fatal infection was invariably produced. Scant numbers of the trypanosomes appeared in the circulating blood of the mice on the first and second days of infection, the flagellates increasing progressively and very rapidly until 50 to 200 trypanosomes per high power microscopic field (10 × 45) were observed on the seventh to tenth days. The acute trypanosomiasis ended fatally within eight to 15 days.

Antibiotics. A total of 30 different antibiotics was supplied by various manufacturers and individuals. These antibiotics were numbered consecutively from 1 to 30 in order to simplify reference. (See Tables 1 and 2.) The sources of these antibiotics are as follows: Abbott Laboratories—#2; Bristol Laboratories—#7; Commercial Solvents Corporation—#12, #19; Dr. J. W. Foster, University of Texas—#4; Heyden Chemical Co.—#23; Hoffman-LaRoche, Inc.—#6; Imperial Chemical Industries, Ltd.—#30; Dr. E. F. Jansen, Western Regional Research Laboratory—#24; Lederle Laboratories—#3; Merck & Co., Inc.—#17; Parke, Davis & Co.—#8, #28; Chas. Pfizer & Co., Inc.—#21, #22, #25, #26, #29; Sharp and Dohme—#20; E. R. Squibb & Sons—#14; The Upjohn Co.—#1, #5, #9, #11, #13, #16, #18; Wallerstein Co., Inc.—#15, #27; Wyeth, Inc.—#10.

In vitro *tests. Tr. cruzi* Pack-Brazil-46 and *Tr. cruzi* WBHT strains were grown at 25°C. in a liquid medium similar to that described by Chang (1947). Seven-day old cultures were used for testing the trypanocidal or trypanostatic effects of the antibiotics.

TABLE 1

The effects of thirty antibiotics on Trypanosoma cruzi in vitro

LOCAL NO.	NAME OF ANTIBIOTIC	DILUTION		THE DEGREE OF MOTILITY OF *Tr. cruzi* CULTURES AFTER EXPOSURE TO A GIVEN ANTIBIOTIC AT THE END OF VARYING TIME INTERVALS				
		In 2% gum acacia or H_2O	Appearance	5 min.	10 min.	15 min.	60 min.	24 hr.
1	Acti-dione	1:10	Susp.	++	+	±	−	−
		1:20	Susp.	+++	++	++	+	−
		1:40	Susp.	+++	+++	+++	++	−
		1:80	Susp.	++++	++++	++++	++++	++++
2	Antibiotic S	1:2,560	Sol.	−	−	−	−	−
		1:5,120	Sol.	±	±	−	−	−
		1:10,240	Sol.	++	+	+	−	−
		1:40,960	Sol.	++++	++++	++++	+	−
		1:81,920	Sol.	++++	++++	++++	++++	++
3	Aureomycin HCl	1:40	Susp.	−	−	−	−	−
		1:80	Susp.	+++	++	±	±	−
		1:160	Susp.	++++	++++	++++	+++	−
		1:320	Susp.	++++	++++	++++	++++	±
4	Bacillin culture filtrate	1:2	Sol.	++++	++++	++++	++++	++++
5	Bacitracin-topical	1:20	Sol.	++++	++++	++++	++++	++
6	Borrelidin sodium	1:8,000	Sol.	++++	++++	++++	++++	++++
7	Bristol A6786-52	1:40	Sol.	++++	++++	++++	++++	++++
8	Chloromycetin (chloramphenicol)	1:10	Susp.	++++	++++	++++	++++	−
		1:80	Susp.	++++	++++	++++	++++	−
		1:160	Susp.	++++	++++	++++	++++	±
9	Circulin (Q-19)	1:20	Sol.	++++	++++	++++	++	±
10	Dihydrostreptomycin sulfate	1:10	Sol.	++++	++++	++++	++++	++++
11	Endomycin	1:6,400	Sol.	−	−	−	−	−
		1:12,800	Sol.	±	±	−	−	−
		1:25,600	Sol.	+++	++	++	++	−
		1:51,200	Sol.	++++	++++	++++	++++	++
12	Fradicin	1:100,000	Alc. sol.	−	−	−	−	−
		1:200,000		±	−	−	−	−
		1:400,000		+	±	±	−	−
		1:800,000		+	±	±	±	±
13	Fumagillin	1:10	Susp.	++++	++++	++++	++++	++++
14	Gliotoxin	1:200	Susp.	++++	+++	+++	−	−
		1:1,600	Susp.	++++	++++	++++	−	−
		1:3,200	Susp.	++++	++++	++++	−	−
		1:6,400	Susp.	++++	++++	++++	±	−
		1:25,600		++++	++++	++++	++++	+++
15	Gramicidin	1:400	Susp.	++++	++++	++++	++++	++++
16	Neomycin sulfate	1:10	Sol.	++++	++++	++++	++++	++++
17	Penicillin G sodium	1:10	Sol.	+++	++	+	+	−
		1:20	Sol.	++++	++++	++++	+++	+

TABLE 1—*Continued*

LOCAL NO.	NAME OF ANTIBIOTIC	DILUTION		THE DEGREE OF MOTILITY OF *Tr. cruzi* CULTURES AFTER EXPOSURE TO A GIVEN ANTIBIOTIC AT THE END OF VARYING TIME INTERVALS.				
		In 2% gum acacia or H_2O	Appearance	5 min.	10 min.	15 min.	60 min.	24 hr.
18	Penicillin O potassium	1:200	Sol.	++++	++++	++++	++++	++++
19	Penicillin, 1,2-diphenyl-2 methyl-amine ethanol	1:200	Susp.	++++	++++	++++	++++	++++
20	Pleocidin	1:10	Sol.	−	−	−	++	+++
		1:20	Sol.	+++	+++	+++	++++	++++
21	Polymyxin B sulfate	1:20	Sol.	++++	+++	+++	++	−
		1:40	Sol.	++++	++++	++++	+++	−
		1:80	Sol.	++++	++++	++++	++++	++
22	Rimocidin	1:64,000	Susp.	−	−	−	−	−
		1:128,000	Susp.	±	±	−	−	−
		1:256,000	Susp.	++	++	±	−	−
		1:512,000	Susp.	++++	++++	++	+	±
23	Streptomycin sulfate	1:10	Sol.	+++	+++	+++	+++	+++
24	Subtilin	1:200	Sol.	++++	+++	++	+	±
25	Terramycin hydro-chloride	1:40	Susp.	−	−	−	−	−
		1:80	Susp.	+++	+++	++	+	−
		1:160	Susp.	++++	++++	++++	++++	++++
	Terramycin hydrochlo-ride + sodium glycinate	1:20	Sol.	±	±	±	−	−
		1:40	Sol.	++	++	++	++	−
		1:80	Sol.	++++	++++	++++	+++	+
26	Thiolutin	1:20	Susp.	++++	++++	++++	++++	−
		1:4,000	Susp.	++++	++++	++++	++++	±
		1:32,000	Susp.	++++	++++	++++	++++	+
		1:128,000	Susp.	++++	++++	++++	++++	++
		1:200,000	Alc. sol.	++++	++++	++++	++++	±
27	Tyrocidine hydro-chloride	1:4,000	Susp.	−	−	−	−	−
		1:8,000	Susp.	±	−	−	−	−
		1:16,000	Susp.	++	++	+	+	−
		1:32,000		++++	++++	++++	+++	++
28	Tyrothricin	1:2,000	Susp.	−	−	−	−	−
		1:4,000	Susp.	±	±	−	−	−
		1:8,000	Susp.	+	+	±	±	−
		1.16,000	Susp.	++	++	++	++	±
29	Viomycin sulfate	1:10	Sol.	++++	++++	++++	++++	++++
30	Viridin	1:200	Susp.	−	−	−	−	−
		1:400	Susp.	±	−	−	−	−
		1:800	Susp.	+	±	−	−	−
		1:1,600	Susp.	+	+	−	−	−
		1:3,200	Susp.	+++	+	+	−	−
		1:6,400		+++	++	++	±	−
		1:12,800		+++	+++	+++	+++	+++

TABLE 1—*Continued*

LOCAL NO.	NAME OF ANTIBIOTIC	DILUTION		THE DEGREE OF MOTILITP OF *Tr. cruzi* CULTURES AFTER EXPOSURE TO A GIVEN ANTIBIOTIC AT THE END OF VARYING TIME INTERVALS.				
		In 2% gum acacia or H_2O	Appearance	5 min.	10 min.	15 min.	60 min.	24 hr.
	Phenol control	1:200	Sol.	−	−	−	−	−
		1:260	Sol.	±	−	−	−	−
		1:300	Sol.	+	+	±	±	−
		1:320	Sol.	++	+	+	±	−
		1:400	Sol.	+++	+++	++	+	−
		1:640	Sol.	++++	++++	++++	++++	±
		1:1,280	Sol.	++++	++++	++++	++++	++
		1:2,560	Sol.	++++	++++	++++	++++	++++
	Merthiolate control	1:200,000	Sol.	−	−	−	−	−
		1:300,000	Sol.	±	−	−	−	−
		1:400,000	Sol.	++	+	±	±	−
		1:600,000	Sol.	+++	++	+	±	±
		1:800,000	Sol.	++++	++++	++++	++++	++++
	Culture + dist. H_2O	1:1		++++	++++	++++	++++	++++
	Culture + 0.9% NaCl sol.	1:1		++++	++++	++++	++++	++++
	Culture in 2% methyl alcohol	1:1		++++	++++	++++	++++	++++
	Culture in 2% ethyl alcohol	1:1		++++	++++	++++	++++	++++

Each antibiotic was tested in as high a concentration as practical from the standpoint of its solubility and availability. Out of 30 antibiotics, 16 were soluble in water, two in alcohol and the rest were used in suspensions of two per cent gum acacia (see Table 1). When alcohol was used in preparing the stock solutions, control tests were run with the alcohol alone to show that, in the concentrations used, those solvents had no immobilizing effect on the trypanosomes.

Kohlmer tubes 85 x 15 mm. were set up with serial dilutions of the antibiotic in 0.5 ml. amounts. *Tr. cruzi* culture in 0.5 ml. amounts was added to all dilutions, the tubes shaken by hand for a few seconds and incubated at 25°C. No bacterial contamination was encountered, although nonsterile technique was used and the tubes unplugged. Phenol and merthiolate were used as controls and for comparison of the trypanostatic and trypanocidal effects of each antibiotic. The pH of each dilution was determined electrometrically and by filter paper. Routine and special controls were set whenever necessary to determine the effect of pH, water, or other solvents on the motility of *Tr. cruzi*.

Timing of the test was started when the trypanosomes were added to the first tube in the series, with readings at 5, 10, 15, and 60 minutes and 24 hours. Observations of the effectiveness of the antibiotic were made by placing a loopful (4 mm. in diameter) of each dilution upon a slide and examining the material without cover glass under low power (10 × 10).

The presence or absence and the degree of motility of trypanosomes per microscopic field were recorded as follows: four plus (++++), 75 to 100 per cent of the trypanosomes motile; three plus (+++), 55 to 75 per cent motile; two plus (++), 15 to 20 per cent motile; one plus (+), five to ten per cent motile; plus-minus (±), one or two sluggish but motile trypanosomes; minus (−), no motile trypanosomes.

In order to determine whether the immobilizing effect of certain drugs upon *Tr. cruzi in vitro* was trypanocidal or trypanostatic, the following procedure was used with two chemicals, Antibiotic S and phenol. The test was carried out as described above, except under aseptic precautions in sterile test tubes. Each tube showing complete immobility was centrifuged and the sediment, representing trypanosomes, was washed three times in Ringer's solution. The packed, washed trypanosomes were resuspended in 1.0 ml. of fresh, liquid trypanosome medium and inoculated into several blood agar slants. These tubes were examined microscopically for growth and motility of *Tr. cruzi* after 10 to 15 days' incubation.

In vivo *tests.* The maximum tolerated dose of each antibiotic was determined when given intraperitoneally to normal mice (*Mus musculus*). However, this dosage in the cases of borrelidin, chloramphenicol, fradicin, gliotoxin, and other antibiotics, proved toxic to infected mice and was reduced to one half when repeating the therapeutic test.

In each series of experiments, 40 to 100 mice, weighing from 18 to 24 grams each, were inoculated with a fatal dose of *Tr. cruzi* (WBHT strain). Microscopic examinations under high power (45 × 10) were made on drops of the tail blood of the mice each day following inoculation. Observations of trypanosomes were recorded as the average number present per microscopic field after a representative number of fields had been noted.

Chemotherapy was begun two to 11 days following infection, when the circulating blood of the mouse contained from two to 25 trypanosomes per microscopic field. All antibiotics were injected intraperitoneally in appropriate amounts in 0.5 ml. of solvent. The screening test consisted of from three to six infected mice for each antibiotic, some of which were repeated several times.

EXPERIMENTAL DATA

Effects of antibiotics on Tr. cruzi in vitro. The 30 drugs tested fell into three groups on the basis of their action; those causing pronounced immobilizing effects, those causing moderate immobilization and those having no effect.

Of the 30 antibiotics tested, nine were found to have pronounced immobilizing effects on *Trypanosoma cruzi in vitro* (see Table 1). All of these nine antibiotics in the following dilutions immobilized the flagellates at the end of five minutes' contact: antibiotic S 1:2,560; aureomycin hydrochloride 1:40; endomycin 1:6,400; fradicin 1:100,000; rimocidin 1:64,000; terramycin hydrochloride 1:40; tyrocidine hydrochloride 1:4,000; tyrothricin 1:2,000; and viridin 1:200. The immobilizing or trypanocidal effect of these antibiotics increased as the contact or exposure time was increased. At the end of 24 hours' exposure, some of these

chemicals were found effective in dilutions as high as 1:40,960 to 1:400,000. Of this group, aureomycin and terramycin had the least immobilizing effect and fradicin the greatest.

The second group of nine antibiotics was found to have a moderate immobilizing effect on the trypanosomes. These antibiotics in the following dilutions were effective after 24 hours' exposure period: acti-dione 1:40; chloramphenicol 1:80; circulin (Q-19) 1:20; gliotoxin 1:6,400; penicillin G sodium 1:10; polymyxin B sulfate 1:40; subtilin 1:200; thiolutin 1:200,000. Some of the above antibiotics showed immobilizing effects as early as 10, 15, or 60 minutes, when used in higher concentrations. Pleocidin, in dilution of 1:10, was effective at 5, 10, and 15 minutes, but lost its immobilizing effect after 60 minutes. (See Table 1.)

The third group consisting of 12 antibiotics, in the highest practical concentrations, was found to have no immoblizing effect on *Tr. cruzi in vitro*. These 12 antibiotics, in the following concentrations, were all found to be without effect at the end of 24 hours' exposure: bacillin culture filtrate 1:2; bacitracin—topical 1:20; borrelidin 1:8,000; Bristol A 6786-52 1:40; dihydrostreptomycin sulfate 1:10; fumagillin 1:10; gramicidin 1:400; neomycin sulfate 1:10; penicillin O potassium (allymercaptomethyl) 1:200; penicillin 1,2-diphenyl-2-methylamine ethanol 1:200; streptomycin sulfate 1:10; Viomycin sulfate 1:10.

Attempts to subculture trypanosomes which were immobile after 24 hours' exposure, as a result of contact with a given dilution of effective antibiotic, yielded negative results.

The addition of an equal volume of hydrochloric acid or sodium hydroxide solution ranging from pH 2.0 to pH 12.0 to trypanosome liquid culture changed the pH of the test culture but did not affect the motility of the trypanosomes after 24 hours' exposure. The flagellates were likewise motile when equal amounts of *Tr. cruzi* culture and water or 0.9 per cent sodium chloride were exposed together for 24 hours.

Merthiolate in dilution of 1:200,000 immobilized the flagellates at the end of 5 minutes, and in dilution of 1:400,000 at the end of 24 hours. These findings were constant in 40 consecutive tests.

Phenol in dilutions up to 1:200 always immobilized *Tr. cruzi* at the end of five minutes, and in dilutions of 1:400 at the end of 24 hours. No efforts were made to determine the "phenol coefficient" of these antibiotics, although it can readily be deduced from the experimental data. (See Table 1.) Antibiotic S, for example, has a phenol coefficient of approximately 10.

No significant difference was observed between the *in vitro* responses of the WBHT and the Pack-Brazil-46 strains of *Tr. cruzi*.

Effects of antibiotics on Tr. cruzi *in vivo*. The sixty-eight untreated control mice (*Mus musculus*) which were inoculated with *Tr. cruzi* WBHT strain all died by acute trypanosomiasis within 16 days. Trypanosomes appeared in the circulating blood of these animals within 24 to 48 hours, the number increasing progressively and rapidly, until the blood was literally swarming with flagellates. Thirty to 100+ parasites per high power microscopic field were usually observed in the blood of infected mice a few days prior to death.

TABLE 2

Treatment of acute cases of experimental Chagas' disease in mice (Mus musculus) with thirty antibiotics

LOCAL NO.	NAME OF ANTIBIOTIC	TREATMENT				
		No. Mice Used	Dosage mg. per Injection	No. Inject. 12-24 hr. Intervals	Death in Days following Infection	Conclusions
1	Acti-dione	6	0.25–0.5	2–5	13–16	Ineffective
2	Antibiotic S	14	1.25–5.0	2–30	7–26	Sl. suppressed & toxic
3	Aureomycin HCl	6	4.0	6–11	11–15	Ineffective
4	Bacillin culture filtrate	3	500.0	3	11–15	Ineffective
5	Bacitracin—topical	3	1.0	6–12	11–21	Ineffective
6	Borrelidin sodium	6	0.02–0.1	9–12	5–7	Ineffective & toxic
7	Bristol A6786-52	4	0.5–1.0	11–16	6–10	Ineffective
8	Chloromycetin (chloramphenicol)	14	2.0–20.0	4–18	5–17	Ineffective
9	Circulin (Q-19)	4	0.1–0.2	10–16	6–10	Ineffective
10	Dihydrostreptomycin sulfate	3	2.0	8–10	13–14	Ineffective
11	Endomycin	4	0.1–0.2	14–30	9–28	Sl. suppressed
12	Fradicin	3	0.005–0.01	10–14	6–9	Ineffective & toxic
13	Fumagillin	3	10.0–20.0	11–13	7–8	Ineffective
14	Gliotoxin	3	0.05–0.1	8–10	5–6	Ineffective & toxic
15	Gramicidin	3	0.025–0.5	27–30	11–26	Sl. suppressed & toxic
16	Neomycin sulfate	4	2.0–0.4	3–12	10–16	Ineffective
17	Penicillin G sodium	5	10.0–30.0	2–13	8–12	Ineffective
18	Penicillin O potassium	4	5.0–10.0	14	10–11	Ineffective
19	Penicillin, 1,2-diphenyl-2 methylamine ethanol	3	5.0–10.0	16–18	10–11	Ineffective
20	Pleocidin	4	0.01–0.1	4–18	7–11	Ineffective
21	Polymyxin B sulfate	3	0.5	6–8	11–12	Ineffective & toxic
22	Rimocidin	5	0.01–0.2	8–30	6–12	Ineffective & sl. suppressed
23	Streptomycin sulfate	3	5.0–10.0	14–16	9–10	Ineffective
24	Subtilin	3	2.5	5–6	7	Ineffective & toxic
25	Terramycin hydrochloride	7	2.0–4.0	6–21	10–15	Ineffective & toxic
26	Thiolutin	2	0.005	12–27	9–19	Sl. suppressed & toxic
27	Tyrocidine hydrochloride	4	0.1–0.2	14–21	9–14	Ineffective
28	Tyrothricin	1	0.05–0.1	14	9	Sl. suppressed & toxic
29	Viomycin sulfate	3	10.0	13	8	Ineffective
30	Viridin	3	0.01–0.1	13	7	Sl. suppressed & toxic
Control infected mice		40	0	0	7–16	Fatality nearly 100%
Control non-infected mice		60	0	0	None	Normal

REMARKS: The following 16 antibiotics in the dosage used all were in solution, i.e., 1, 3, 4, 5, 6, 7, 8, 9, 10, 15, 16, 17, 19, 20, 22 and 23. The remaining antibiotics used were in fine suspension of 2% gum acacia.

The following 23 antibiotics, in the dosages listed in Table 2, were without any curative effect upon Chagas' disease in *Mus musculus* (three to six mice were tested with each antibiotic): acti-dione, aureomycin hydrochloride, bacillin culture filtrate, bacitracin, borrelidin sodium, Bristol A6786-52, chloramphenicol, circulin, dihydrostreptomycin sulfate, fradicin, fumagillin, gliotoxin, neomycin sulfate, penicillin G sodium, penicillin O potassium, 1,2-diphenyl-2-methylamine ethanol penicillin, pleocidin, polymyxin B sulfate, streptomycin sulfate, subtilin, terramycin hydrochloride (also terramycin hydrochloride + sodium glycinate), tyrocidine hydrochloride, and viomycin sulfate. All the experimental animals died from acute trypanosomiasis, like the control animals, within two weeks.

A few instances of suppressive action were obtained when mice infected with Chagas' disease were treated intraperitoneally with certain dosages of the following antibiotics: antibiotic S, endomycin, gramicidin, rimocidin, thiolutin, tyrothricin, and viridin. Although there were definite reductions in the number of trypanosomes present in the blood of these mice as compared to the number in the blood of untreated mice, many died earlier than did the controls because of the toxicity of the drugs. In the three which survived after treatment with antibiotic S, endomycin and gramicidin, the infection was suppressed from an acute, fatal one to a chronic form. Those treated with endomycin (30 doses of 0.2 mg. each) and gramicidin (30 doses of 0.5 mg. each) showed pronounced edema and both died after 21 days, apparently from toxicity of the drug. The remaining mouse, which had received 30 doses of 1.25 mg. each of antibiotic S, was sacrificed after 40 days, and culture tests showed trypanosomes present in the blood, indicating that no absolute cure was obtained.

DISCUSSION

The evaluation of drugs against Chagas' disease has been difficult in the past because *Tr. cruzi* infections in experimental animals run a chronic course, and only scant numbers of parasites are detectable in the circulating blood of the host. However, the exalted and virulent strains of *Tr. cruzi*, such as WBHT and "Tulahuen," the Chilean strain, which produce acute and fatal infection in mice (*Mus musculus*), now make the experimental chemotherapy relatively simple.

One of the remarkable features of this exalted strain of *Tr. cruzi* is that it will invariably produce fatal infection within two weeks, and that the trypanosomes in the circulating blood of the mice increase rapidly to the same height as in *Tr. brucei* or *Tr. equiperdum* infections.

The absolute identification of the WBH strain used in this study as *Tr. cruzi* was established by passage into *Triatoma gerstaeckeri*, one of the natural vectors of this parasite. The flagellates in the insects multiplied in the manner characteristic of *Tr. cruzi*. After a few months, the insects were crushed and inoculated into normal mice, all of which contracted the infection. From the mice, the *Tr. cruzi* was recultured on Novy and MacNeal's medium and designated as the WBHT strain. This strain killed *Mus musculus* within two weeks.

Although *in vitro* effects were obtained against *Tr. cruzi* with 18 of the 30 antibiotics used in this study, none of these drugs yielded curative effects when

Mus musculus, infected with acute Chagas' disease, were subjected to chemotherapy. A few antibiotics showed a suppressive effect, which suggests that increased dosage, more frequent administration, or combination with other drugs might produce more promising results.

The *in vitro* effects of pleocidin are rather interesting. This antibiotic, in dilution of 1:10, immobilized *Tr. cruzi* at the end of five minutes. However, after 60 minutes of contact, the flagellates regained their motility. This temporary "antiseptic effect" is probably due to instability of the antibiotic or chemical change of the antibiotic after prolonged contact with the trypanosome culture.

When *Tr. cruzi* were rendered immobile *in vitro* with other agents, including the control chemicals, merthiolate and phenol, this activity was parallel with trypanocidal effect. The washed trypanosomes, when introduced into suitable culture media, failed to show motility or to multiply.

Although some of the antibiotics had a very pronounced effect *in vitro*, they failed to prove effective *in vivo*. In contrast to these findings, other drugs tested by Packchanian (1952) have yielded suppressive effects *in vivo* but were without effect *in vitro*.

SUMMARY

The following antibiotics were tested against *Trypanosoma cruzi* both *in vitro* and *in vivo*, 23 for the first time: 1. acti-dione; 2. antibiotic S; 3. aureomycin hydrochloride; 4. bacillin culture filtrate; 5. bacitracin—topical; 6. borrelidin sodium; 7. Bristol A6786-52; 8. chloramphenicol (chloromycetin); 9. circulin (antibiotic Q-19); 10. dihydrostreptomycin sulfate; 11. endomycin; 12. fradicin; 13. fumagillin; 14. gliotoxin; 15. gramicidin; 16. neomycin sulfate; 17. penicillin G sodium; 18. penicillin O potassium (allymercaptomethyl); 19. 1,2-diphenyl-2-methylamine ethanol penicillin; 20. pleocidin; 21. polymyxin B sulfate; 22. rimocidin; 23. streptomycin sulfate; 24. subtilin; 25. terramycin hydrochloride; 26. thiolutin; 27. tyrocidine hydrochloride; 28. tyrothricin; 29. viomycin sulfate; 30. viridin.

In vitro tests were performed by a newly developed method with the 30 antibiotics against two strains of *Tr. cruzi* (Pack-Brazil-46 and WBHT). Out of the 30 antibiotics, 18 showed immobilizing or trypanocidal effects at the end of 5, 10, 15, and 60 minutes or at the end of 24 hours. The trypanostatic or trypanocidal effects ranged from dilutions of 1:10 to as high as dilutions 1:400,000, depending upon the antibiotics and time of contact periods. The remaining 12 antibiotics (Nos. 4, 5, 6, 7, 10, 13, 15, 16, 18, 19, 23, 29), used in highest concentrations practical, were innocuous to *Tr. cruzi in vitro* at the end of 24 hours' exposure period.

In vivo tests, in spite of the above findings, were all essentially negative. None of the above 30 antibiotics, when administered intraperitoneally in maximum tolerated doses into mice infected with the virulent WBHT strain of *Tr. cruzi*, produced an absolute cure. All of the experimental animals, with the exception of a few which developed chronic infections, died of acute trypanosomiasis within two weeks. A few instances of suppressive action were obtained with the

following antibiotics: endomycin, gramicidin, rimocidin, antibiotic S, thiolutin, tyrocidine hydrochloride, tyrothricin, and viridin. Although there were definite reductions in the number of trypanosomes present in the blood of these mice as compared with the number in the untreated mice, many died earlier than did the controls because of the toxicity of the drugs. The most marked suppression was produced by antibiotic S. However, no curative effects were obtained.

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EXPERIMENTAL CHEMOTHERAPY OF TRYPANOSOMIASIS

III. Effect of Achromycin[1] Against *Trypanosoma Equiperdum* and *Trypanosoma cruzi*

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INTRODUCTION

During an antibiotic screening program in cooperation with our Mycology Department for new substances which might be effective against animal parasites, it has been found that an antibiotic obtained from a new species of actinomycete, *Streptomyces albo-niger*, is active *in vivo* against *Trypanosoma equiperdum* and is partially effective against *T. cruzi in vivo* and *in vitro*. This has been given the name Achromycin, and its physical characteristics and *in vitro* spectrum have been described in a previous paper from these laboratories by Porter *et al.* (1952).

The majority of published results obtained with antibiotics in trypanosomiasis have not been encouraging. As early as 1935, Fischl claimed that prodigiosin perchlorate, obtained from *Bacillus prodigiosus*, produced a "definite" effect against *T. brucei* in mice when administered subcutaneously in oil at a dose of 500 mg. per kg. Rubin (1947), however, found prodigiosin tartrate to be ineffective *in vivo* at this dosage. Waksman *et al.* (1941) and Robinson (1943) believed that streptothricin and actinomycin prolonged the lives of mice infected with *T. equiperdum*, but cures were not claimed. Schatz *et al.* (1946) isolated a fungus by a method of soil enrichment which was trypanocidal *in vitro* but had no *in vivo* effects against *T. equiperdum*. Penicillin has not proven effective against *T. equiperdum* (Neghme 1945) or *T. rhodesiense* (Nelson, 1945) *in vivo*, although Torrealba (1945) and Earle (1946) believe that it may be of value in Chagas' disease. Anderson *et al.* (1946) stated that subtilin caused lysis of *T. equiperdum in vitro*, but was without effect *in vivo*. Aureomycin, chloramphenicol, streptomycin, subtilin, bacitracin and clavacin have produced no effects against *T. equiperdum* in these laboratories, even when infected mice were given excessive doses orally or parenterally several times daily until the crisis was reached. Cosar *et al.* (1952) have demonstrated recently that a crystalline antibiotic obtained from a species of *Streptomyces* is effective against *T. congolense* in mice when given in a single subcutaneous dose of 150 mg. per kg. Their follow-up period after treatment, however, extended for only 12 days, so that it has not been demonstrated that this substance is curative.

In view of the unsuccessful attempts with other antibiotics to cure trypanosome infections in mammalian hosts, we believe that Achromycin is unique among crystalline substances isolated thus far from fungi or bacteria, because in addition to a fairly broad *in vivo* spectrum against bacteria (Porter *et al.*, 1952) it will also

[1] Trade mark for an antibiotic produced from a species of Actinomyces—*Streptomyces albo-niger*.

cure *T. equiperdum* infections in mice and rabbits and is partially effective against *T. cruzi*.

METHODS

T. equiperdum in mice. The methods used in our assays for activity against *T. equiperdum* in mice have been described in detail in a previous publication (Hewitt *et al.*, 1949). The strain of *T. equiperdum* used was obtained originally through the kindness of Dr. A. L. Tatum, University of Wisconsin, in 1945, and has been maintained by serial transfers every three days in mice. For routine assays, mice were inoculated with a saline suspension containing approximately 50,000 to 100,000 trypanosomes in 0.2 cc. This produced highly consistent infections in untreated mice and death occurred invariably within 3 to 5 days after inoculation. When treated mice survived beyond the period when all controls were dead, activity was indicated. The following definition of terms is given as they are used in this paper:

Suppressive effect. Treated mice lived for varying periods of time after all controls were dead, but less than 50 per cent survived a 30-day observation period after cessation of treatment.

Curative effect. Fifty per cent or more treated mice survived for 30 days after cessation of treatment.

Curative dose (CD_{50}). The least dose needed to produce a curative effect.

Lethal dose (LD_{50}). The least dose needed to kill 50 per cent or more mice within 24 hours after a single dose, or on the first or second day of multiple dosage when multiple dosage was used.

Since the determination of curative doses by any route of administration is affected by the number of trypanosomes inoculated, the intensity of infection when treatment is begun and the frequency of treatment, we have used both multiple and single dosage in our studies with Achromycin, and have administered dosage by several different routes or at different periods during the course of the infection. The hydrochloride salt was used in all the assays described herein. This is freely soluble in water and the pH was not adjusted before administration.

T. equiperdum in rabbits. T. equiperdum produces chronic infections in rabbits, characterized by edema and inflammation of the ocular, nasal, oral and perianal regions, skin lesions, conjunctivitis, blepharitis and rhinitis. Death usually occurs within 2 to 3 months after inoculation, and trypanosomes are seldom found in large numbers in the circulating blood. Symptoms generally appear within 2 weeks after inoculation and become progressively worse until death occurs.

In our experiments, mature rabbits of both sexes and mixed varieties, weighing from 2 to 3.5 kilos, were inoculated intravenously with saline suspensions containing from 500,000 to 1,000,000 *T. equiperdum* obtained from mouse donors. Treatment with Achromycin was administered intravenously or intramuscularly at various periods after inoculation. Pretreatment lesions were recorded or photographed, and post-treatment observations were continued for from 3 to 6 months after treatment was stopped. Untreated controls usually displayed lesions within 10 days after inoculation and generally died within 30 to 90 days. In treated

rabbits, survival for 3 to 6 months, regression of pretreatment lesions with no demonstrable residual involvement and noninfectivity of blood from treated rabbits to clean rabbits and mice were taken as criteria of cure.

T. cruzi in vitro. The liquid media described by Sampath and Little (1949) and Little and SubbaRow (1945) were used for *in vitro* assays of Achromycin against *T. cruzi*. Counted numbers of trypanosomes were introduced into media containing various amounts of Achromycin and these were incubated at 28° to 32° C. The cultures were examined and trypanosomes were counted at various intervals during exposure to Achromycin. The strain of *T. cruzi* used was the same as for the *in vivo* studies described below.

T. cruzi in vivo. Mice weighing from 15 to 25 grams were inoculated subcutaneously with 0.2 cc. of a saline suspension containing approximately 500,000 to 1,000,000 *T. cruzi*. The strain used (B) was obtained in 1948 through the courtesy of Dr. F. J. Brady, National Institutes of Health. From 60 to 80 per cent of untreated mice died within 2 weeks after inoculation, and from 90 to 100 per cent were dead within 4 weeks. Treatment with Achromycin was administered orally or parenterally, usually in multiple doses over a period of 1 to 2 weeks, beginning on various days after inoculation. The criterion of "cure" was survival of 50 per cent or more of the treated mice for 30 days after cessation of treatment.

EFFECT OF ACHROMYCIN AGAINST *T. EQUIPERDUM* IN MICE

When administered intraperitoneally twice daily for 7 doses, starting 2 to 6 hours after inoculation, a total dosage of from 70 to 140 mg. of Achromycin per kg. (10 to 20 mg. per kg. per dose) has generally been required to produce a curative effect. This has varied with different batches submitted for assay, and in different groups of animals treated with the same batch. Curative effects have been obtained with total doses as low as 56 mg. per kg. (8 mg. per kg. for 7 doses), but in some experiments all mice died within 30 days after inoculation following total doses of from 210 to 280 mg. per kg. (30 mg. to 40 mg. per kg. for 7 doses). We believe that concurrent bacterial or virus infections were responsible for deaths in the latter cases, however, since trypanosomes could not be found in tail blood shortly before death and total doses of 280 mg. per kg. had not been found to be toxic.

When treatment was withheld for 24 to 48 hours after inoculation, the intraperitoneal dose of Achromycin required to produce a curative effect was correspondingly greater. Total doses of less than 105 mg. per kg. (15 mg. per kg. for 7 doses) have rarely been curative when treatment was started 24 hours after inoculation, and at least 280 mg. per kg. (40 mg. per kg. for 7 doses) has been necessary to produce cures when treatment was withheld for 48 hours after inoculation. Table 1 presents data from mice treated with various doses when treatment was started at 4, 24 and 48 hours after inoculation. The effect of a single intraperitoneal dose of 75 mg. per kg. of Achromycin, administered 24 hours after inoculation with varying numbers of trypanosomes, is given in Table 2. Single doses of less than 100 mg. per kg. have generally failed to produce curative effects when 50,000 or more trypanosomes were inoculated, but as demonstrated

in Table 2 there was a positive correlation in this respect between the number of trypanosomes used for inoculum and the dosage of Achromycin administered.

TABLE 1

Effect of Achromycin against Trypanosoma equiperdum *in mice when treatment was started at different periods during the course of the infection*

DOSAGE MG./KG. (IP.)	TIME TREATMENT STARTED (AFTER INOCULATION)	DEATHS—WEEKS AFTER INOCULATION (10 MICE IN EACH GROUP)				PER CENT SURVIVALS 30 DAYS AFTER INOCULATION	ACTIVITY
		1	2	3	4		
5 BID 7 doses	4 hrs.	0	9	1	—	0	Suppressive
5 BID 7 doses	24 hrs.	3	7	—	—	0	Suppressive
5 BID 7 doses	48 hrs.	10	—	—	—	0	Negative
10 BID 7 doses	4 hrs.	1	3	0	0	60	Curative
10 BID 7 doses	24 hrs.	1	7	1	0	10	Suppressive
10 BID 7 doses	48 hrs.	0	8	2	—	0	Suppressive
15 BID 7 doses	4 hrs.	0	3	0	0	70	Curative
15 BID 7 doses	24 hrs.	0	1	3	0	60	Curative
15 BID 7 doses	48 hrs.	3	2	4	0	10	Suppressive
20 BID 7 doses	4 hrs.	0	0	2	1	70	Curative
20 BID 7 doses	24 hrs.	0	1	1	1	70	Curative
20 BID 7 doses	48 hrs.	4	0	3	0	30	Suppressive
40 BID 7 doses	4 hrs.	0	0	1	0	90	Curative
40 BID 7 doses	24 hrs.	1	0	1	1	70	Curative
40 BID 7 doses	48 hrs.	0	1	0	1	80	Curative
Controls		10	—	—	—	0	—

TABLE 2

Effect of a single dose of Achromycin against Trypanosoma equiperdum *in mice following different amounts of inoculum*

NO. OF TRYPANOSOMES INOCULATED (PER 20 GM. MOUSE)	DOSAGE MG./KG. IP (ONE DOSE 24 HRS. AFTER INOCULATION)	DEATHS—WEEKS AFTER INOCULATION (10 MICE IN EACH GROUP)				PER CENT SURVIVALS 30 DAYS AFTER INOCULATION	ACTIVITY
		1	2	3	4		
60,000	75	0	7	0	0	30	Suppressive
60,000	None (controls)	10	—	—	—	0	—
6,000	75	0	3	1	0	60	Curative
6,000	None (controls)	10	—	—	—	0	—
600	75	0	4	0	0	60	Curative
600	None (controls)	10	—	—	—	0	—
60	75	0	0	0	0	100	Curative
60	None (controls)	10	—	—	—	0	—
6	75	0	0	1	0	90	Curative
6	None (controls)	3	1	0	0	60*	—

* Due to the very small inoculum all controls did not die in this case.

When Achromycin was administered parenterally to mice near the crisis of infections with *T. equiperdum*, effective doses caused a precipitous drop in the number of trypanosomes and cleared the blood of trypanosomes within 48 hours

(Table 3). Total doses of 380 mg. per kg. (40 mg. per kg. for 7 doses), and single doses of 400 mg. per kg. were curative.

TABLE 3

Effect of Achromycin against Trypanosoma equiperdum *in mice when treatment was started near the crisis of the infection*

DOSAGE (MG./KG. IP.) (TREATMENT STARTED 2 DAYS AFTER INOCULATION)	TOTAL DOSE MG./KG.	AVERAGE NO. TRYPANOSOMES PER 100 OIL IMMERSION FIELDS (DAYS AFTER INOCULATION)				DEATHS—WEEKS AFTER INOCULATION (10 MICE IN EACH GROUP)				PER CENT SURVIVALS 30 DAYS AFTER INOCULATION
		2	3	4	7	1	2	3	4	
2.5 BID 7 doses	17	600	9,378	—	—	10	—	—	—	0
5 BID 7 doses	35	472	4,840	9,127	—	8	2	—	—	0
10 BID 7 doses	70	575	1,480	598	0	0	10	—	—	0
20 BID 7 doses	140	519	825	520	0	0	2	3	2	20
40 BID 7 doses	280	565	409	0	0	0	0	0	0	100
100 BID 1 dose	100	416	155	0	4	0	10	—	—	0
200 BID 1 dose	200	698	34	0	0	0	7	2	0	20
300 BID 1 dose	300	343	11	0	0	0	5	1	0	40
400 BID 1 dose	400	136	1	0	0	1	0	3	0	60
500 BID 1 dose	500	260	0	0	0	4	1	1	3	10*
Controls	—	190	9,290	14,366	—	10	—	—	—	0

* toxic dose.

TABLE 4

Comparison of Achromycin with three other trypanocides when administered orally for 7 doses to mice infected with Trypanosoma equiperdum

COMPOUND	DOSAGE MG./KG ORALLY (TREATMENT STARTED 4 HRS. AFTER INOCULATION)	DEATHS—WEEKS AFTER INOCULATION (10 MICE IN EACH GROUP)				PER CENT SURVIVALS 30 DAYS AFTER INOCULATION	ACTIVITY
		1	2	3	4		
Antrycide	5	0	7	0	0	30	Suppressive
	10	0	5	4	0	10	Suppressive
	15	0	2	3	0	50	Curative
	20	0	1	0	1	80	Curative
Stilbamidine	1.5	4	5	1	—	0	Suppressive
	3	0	2	0	0	80	Curative
	6	0	0	0	0	100	Curative
p-Phenylene diguanidine	6	0	10	—	—	0	Suppressive
	12.5	0	5	1	0	40	Suppressive
	20	0	0	2	3	50	Curative
	30	0	0	0	0	100	Curative
Achromycin	5	0	10	—	—	0	Suppressive
	10	0	9	0	0	10	Suppressive
	20	0	2	1	0	70	Curative
	25	0	1	0	0	90	Curative
Controls	—	10	—	—	—	0	—

Administered orally, Achromycin has produced curative effects against *T. equiperdum* in mice at dosage levels slightly higher than those required when given parenterally. Generally, total oral doses of from 140 to 210 mg. per kg. (20 to

30 mg. per kg. for 7 doses) have been curative when treatment was started 2 to 6 hours after inoculation. A comparison of effective doses of Achromycin and three other trypanocides administered orally in given in Table 4.

In experiments designed to determine the possible trypanocidal versus trypanostatic effect of Achromycin, it was found that blood withdrawn from mice 30 days after termination of treatment and inoculated into fresh mice did not produce trypanosome infections within a 30-day period. When viable trypanosomes were inoculated into formerly trypanosome-infected mice which had lived 30 days after treatment with Achromycin, acute, fulminating infections developed and death occurred within 6 days after inoculation. We believe this denotes that Achromycin is definitely trypanocidal *in vivo*.

Attempts have been made to discover whether *T. equiperdum* will become resistant to Achromycin after repeated exposure to sub-curative doses. These long term experiments have been complicated from time to time, however, by what we believe to be virus or bacterial infections in some of our mice after several transfers of Achromycin-exposed trypanosomes. These experiments are being continued and it has not yet been decided whether drug resistance occurs.

EFFECT OF ACHROMYCIN AGAINST *T. EQUIPERDUM* IN RABBITS

The effects of different doses of Achromycin against *T. equiperdum* in rabbits have varied at different period during the course of the infections. When treatment was started within 4 hours after inoculation, 10 mg. per kg., twice daily, intravenously for 7 days, prevented the appearance of lesions for a three-month period of observation. One-half this dose delayed the appearance of lesions, but generalized infections developed eventually. When treatment was withheld for 10 days, until mild ocular and perianal lesions were displayed, 10 mg. per kg., twice daily, intravenously, for 7 days, caused a regression of lesions but relapse occurred shortly after cessation of treatment. The same dosage given for 14 days was curative, in that the lesions disappeared completely and did not recur during a five-month observation period.

Within from 4 to 6 weeks after inoculation, the lesions in rabbits due to *T. equiperdum* become severe. The eyes, nose, ears, skin and perianal region are involved to various degrees,with considerable inflammation, edema, scabbing and ulceration (see plates). These symptoms are accompanied by a rapid loss in weight. When treatment with Achromycin was begun at this stage of the infection, 20 mg. per kg., once daily, intravenously for from 7 to 14 days (total dosage, 140 to 280 mg. per kg.) cured 8 out of 11 rabbits, and 40 mg. per kg., twice daily for the same period (total dosage 280 to 560 mg. per kg.), cured 6 out of 6 rabbits. Follow up observations after disappearance of the lesions were continued for as long as 6 months after treatment; the rabbits gained weight and remained normal in appearance in most cases.

Intramuscular injections of Achromycin, given on alternate days for 4 to 7 doses, at 20 and 40 mg. per kg., cured 6 out of 8 rabbits when treatment was begun 4 to 5 weeks after inoculation. Dosage was administered in water or sesame oil with the same results. When fewer than 4 intramuscular injections of Achromycin were given, a regression of lesions sometimes occurred but this was always

followed by relapse. The rapidity of disappearance of lesions, even those of severe nature, during and following intramuscular or intravenous treatment with Achromycin, was dramatic. Within one week after the first dosage was administered, swollen and inflamed tissues subsided noticeably, scabbed areas began to slough, nasal congestion cleared up and ulcers healed. Within 10 days to 2 weeks most of the affected areas regained their normal appearance and following curative doses usually remained so throughout the post-treatment observation period. This is illustrated in the accompanying plates.

Blood withdrawn from a rabbit 3 months after treatment with Achromycin was inoculated into clean mice and trypanosomes did not appear within a six week observation period. Of three clean rabbits inoculated with blood from the above donor, two did not display lesions characteristic of trypanosomiasis during a four-month follow up period. The third developed a conjunctivitis with paralysis of the right fore and hind legs 2 months after inoculation. Four rabbits which were considered cured after Achromycin therapy were held for 3 months and then challenged with 0.5 cc. of a heavy inoculum containing *T. equiperdum* from mouse donors. One of these developed severe eye, nose and ear lesions, typical of trypanosomiasis, about 2 months after inoculation. Two others developed lesions on the neck and abdomen which were not typical of primary infections with *T. equiperdum*, but might have been attenuated by a residual immunity. The fourth rabbit displayed no lesions during a four month follow up period after inoculation.

EFFECT OF ACHROMYCIN AGAINST *T. CRUZI*

In vitro experiments. As demonstrated in the accompanying tables, the addition of Achromycin to cultures of *T. cruzi* inhibits growth in dilutions up to 1–40,000. At 1–400,000 a measurable effect has been observed in some protocols, but this has been variable. The data in Table 5 illustrate variation within different experiments relative to the effects of Achromycin. In most cases dilutions of 1–40,000 or less inhibited growth of *T. cruzi* by 80 to 90 per cent when readings were taken after one week's incubation. The effects at 24 hours, 96 hours, and 6 days exposure to Achromycin are given in Table 6, and included in this table is one protocol showing inhibition of growth at 1–10,000, 35 days after subculture. The effects of Achromycin on cultures containing varying amounts of inoculum are given in Table 7. With the exception of group 4, a dilution of 1–40,000 inhibited growth considerably.

The information obtained thus far suggests that the *in vitro* effect of Achromycin on *T. cruzi* is trypanostatic rather than trypanocidal, since rarely has growth been suppressed entirely and subcultures have always been positive.

In vivo experiments. The results obtained with Achromycin *in vivo* against *T. cruzi* have been extremely variable. When treatment was started within 24 hours after inoculation, 20 mg. per kg., twice daily, intraperitoneally, for 12 doses (total dose—220 mg./kg.) has "cured" 50 per cent of the mice in some experiments but has failed in others. Dosages higher than this, administered parenterally or orally, have likewise given erratic results. When treatment was withheld for more than 24 hours, no effects could be determined, and it is assumed, therefore,

TABLE 5

Effect of Achromycin in vitro *on* Trypanosoma cruzi *after incubation for 1 week at 28° to 32° C.*

DILUTION	TRYPANOSOME COUNT PER 0.1 CMM. OF MEDIA								
	Group 1			Group 2			Group 3		
	Initial count = 3			Initial count = 10			Initial count = 23		
	Tube 1	Tube 2	Control	Tube 1	Tube 2	Control	Tube 1	Tube 2	Control
1–200	5	10	50	10	5	80	10	20	1,200
1–400	5	10	40	10	10	75	75	50	1,325
1–4,000	10	15	60	10	10	50	100	135	1,300
1–40,000	10	5	40	35	40	50	340	350	960
1–400,000	15	15	30	45	35	45	1,070	1,100	1,400
	Group 4			Group 5			Group 6*		
	Initial count = 18			Initial count = 18			Initial count = 15		
1–200	25	10	925	35	20	910	—	—	—
1–400	50	35	865	35	25	950	65	75	55,300
1–4,000	80	75	900	85	125	925	140	130	4,000
1–40,000	270	310	950	180	280	870	370	250	4,750
1–400,000	560	510	970	730	800	970	950	750	5,250

* Whole blood added as enrichment to this group.

TABLE 6

Effect of Achromycin in vitro *on* Trypanosoma cruzi *at various times during incubation at 28° to 32° C.*

DILUTION	TRYPANOSOME COUNT PER 0.1 CMM. OF MEDIA											
	Initial count = 415											
	24 hr.			96 hrs.			6 days			35 days after subculture		
	Tube 1	Tube 2	Control	Tube 1	Tube 2	Control	Tube 1	Tube 2	Control	Tube 1	Tube 2	Control
1–1,000	35	30	60	25	40	130	25	40	210	35	75	295
1–10,000	35	35	100	20	30	155	50	20	270	25	90	350
1–100,000	65	70	100	140	85	145	220	145	285	515	180	400

TABLE 7

Effect of Achromycin in vitro *on* Trypanosoma cruzi *in cultures containing different amounts of inoculum after incubation for 1 week at 28° to 32° C.*

DILUTION	TRYPANOSOME COUNT PER 0.1 CMM. OF MEDIA											
	Group 1			Group 2			Group 3			Group 4		
	Initial count 535			Initial count 270			Initial count 135			Initial count 70		
	Tube 1	Tube 2	Control	Tube 1	Tube 2	Control	Tube 1	Tube 2	Control	Tube 1	Tube 2	Control
1–400	35	40	290	15	20	40	10	15	70	15	10	25
1–4,000	60	50	300	10	20	70	5	10	75	15	20	50
1–40,000	60	90	270	40	30	240	0	0	10	10	5	10
1–400,000	240	160	1,000	75	75	175	0	25	45	50	15	25

that in the doses used thus far, Achromycin has no effect on the tissue stages of *T. cruzi* in mice. The effects on circulating trypanosomes, though not constant, are of considerable interest, since few known compounds show any effect whatever upon *T. cruzi* in laboratory animals.

TOXICITY

Preliminary data supplied by Dr. R. W. Cunningham of our pharmacology department give the LD_{50} values of Achromycin in the mouse as 360 mg. per kg. (intravenous), 520 mg. per kg. (intraperitoneal) and 678 mg. per kg. (oral). In rats these figures are somewhat lower. A pathology report from Dr. F. Dessau states that there is evidence of kidney damage after the repeated administration to rats of from 25 to 100 mg. per kg. parenterally for from 1 to 4 weeks. These studies are being extended.

DISCUSSION

Although many types of compounds of widely different chemical structure have been demonstrated to be trypanocidal in laboratory animals, relatively few of these are currently used to treat trypanosomiasis in man or domesticated animals. Moreover, the compounds now used to treat trypanosomiasis all have limitations of one sort or another and there is a recognized need for new types of trypanocidal compounds. The effects produced by Achromycin against *T. equiperdum* in mice and rabbits are of interest in this respect, since antibiotics have previously shown very little promise against trypanosome infections. The effective parenteral dose of Achromycin is far greater than is required with other trypanocidal substances such as Bayer 205, Stilbamidine, Antrycide and the arsenicals. A comparison of the effective dosages of known trypanocides in laboratory hosts can be misleading, however, insofar as possible clinical application is concerned. The single dose technique which has been used commonly for evaluating trypanocidal activity in mice has established standards of comparison which frequently mean very little in clinical application. Bayer 205 (Naphuride sodium, Antrypol, Germanin, etc.), for example, will cure *T. equiperdum* infections in mice at a single parenteral dosage of 0.2 mg. per kg., whereas to produce the same effect with Tryparsamide a dosage of 1000 mg. per kg. is required. In using these compounds for the treatment of human trypanosomiasis, on the other hand, multiple doses are administered and the ratio of total dosage of Bayer 205 to Tryparsamide is about 1:4.

The rate of absorption and excretion of Achromycin is not known, but the data obtained thus far indicate that multiple dosage over a period of several days is more effective against *T. equiperdum* than the single administration of a large dose. Moreover, single doses which produce a curative effect are very close to the toxic dose. It is apparently absorbed quite readily from all tissues, since effective intraperitoneal, intravenous, intramuscular and oral doses are within the same general range. Attempts to delay absorption by using oil as a menstruum have not been successful thus far.

The data obtained from rabbits and mice indicate that toxic doses of Achro-

mycin are several times greater than curative doses. Evidence obtained by our pharmacology and pathology departments relative to delayed toxicity after repeated dosage denotes that this is not an atoxic antibiotic, however, and caution will be necessary in clinical trials.

SUMMARY

The effects of Achromycin, a new antibiotic isolated from *Streptomyces albo-niger*, against *Trypanosoma equiperdum* in mice and rabbits, and *T. cruzi in vitro* and in mice are described. When administered intraperitoneally to mice in multiple doses, beginning within 2 to 6 hours after inoculation with trypanosomes, the total dosage required to cure *T. equiperdum* infections is about 70 to 140 mg. per kg. (10 to 20 mg. per kg. for 7 doses). It is effective orally at slightly higher doses. When treatment is withheld until near the crisis of infections, correspondingly higher doses are required to produce curative effects. Single parenteral doses of 100 mg. per kg. or greater are required to produce cures, although there is a positive correlation in this respect between the number of trypanosomes used for inoculum and the dosage of Achromycin administered. In rabbits, infected with *T. equiperdum*, 20 mg. per kg., once daily, intravenously for from 7 to 14 days (total dosage, 140 to 280 mg. per kg.) has caused the disappearance of well developed lesions with no relapse for 3 to 5 months thereafter. Intramuscular treatment is effective at 40 mg. per kg. for four or more doses. Trypanostatic effects against *T. cruzi in vitro* are produced by Achromycin in dilution of 1–40,000 or less, and variable effects are produced *in vivo* against this parasite in mice when treatment with 20 mg. per kg. or more twice daily, for 12 to 24 doses is administered, starting treatment within 24 hours after inoculation. Some mice have been "cured" at these doses and others have not. Achromycin is apparently not effective against the tissue stages of *T. cruzi*. The LD_{50} values of Achromycin in mice are 360 mg. per kg. (intravenous), 520 mg. per kg. (intraperitoneal) and 678 mg. per kg. (oral), and there is evidence of kidney damage after the repeated administration of 25 to 100 mg. per kg. parenterally to rats for from 1 to 4 weeks.

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PLATE I

FIG. 1. Rabbit 2W. 34 days after inoculation with *T. equiperdum*. Severe ocular and nasal lesions. Treatment with Achromycin started, 20 mg. per kg., intravenously, once daily for 7 days.

FIG. 2. Rabbit 2W. One day after last treatment.

FIG. 3. Rabbit 2W. Three weeks after treatment. No relapse 6 months after treatment.

PLATE II

FIG. 1. Rabbit 5Y. 34 days after inoculation with *T. equiperdum*. Severe ocular and nasal lesions. Treatment with Achromycin started, 40 mg. per kg., intravenously, once daily for 7 days.

FIG. 2. Rabbit 5Y. One day after last treatment.

FIG. 3. Rabbit 5Y. Three weeks after treatment. No relapse 3½ months after treatment. Challenged with fresh inoculum; developed severe facial lesions and died 3 months after second inoculation.

PLATE III

FIG. 1. Rabbit 10Y. 34 days after inoculation with *T. equiperdum*. Severe ocular lesions, with right eye completely obliterated by scab formation. Treatment with Achromycin started, 40 mg. per kg., intramuscularly in sesame oil, on alternate days for 7 doses.

FIG. 2. Rabbit 10Y. One day after the 4th dose.

FIG. 3. Rabbit 10Y. Two weeks after last treatment. Relapsed 3½ months after treatment.

1 2 3

PLATE I

1 2 3

PLATE II

1 2 3

PLATE III

1 2 3

PLATE IV

FIG. 1. Rabbit 3W, 34 days after inoculation with *T. equiperdum*. Severe nasal lesions, mild ocular involvement. Treatment with Achromycin started, 20 mg. per kg., intravenously, once daily for 14 days.

FIG. 2. Rabbit 3W. One day after the 7th dose.

FIG. 3. Rabbit 3W. Two weeks after last treatment. No relapse $3\frac{1}{2}$ months after treatment. Challenged with fresh inoculum; no lesions developed 4 months after second inoculation.

1 2 3

PLATE V

FIG. 1. Rabbit 3Y, 34 days after inoculation with *T. equiperdum*. Severe ocular and ear lesions. Treatment with Achromycin started, 20 mg. per kg., intravenously, once daily for 14 days.

FIG. 2. Rabbit 3Y. One day after the 7th dose.

FIG. 3. Rabbit 3Y. Two weeks after the last treatment. No relapse 6 months after treatment.

ONE METHOD FOR THE PURE CULTURE OF *TRICHOMONAS VAGINALIS*

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While experimental culture of *Trichomonas vaginalis* has been attempted for many years, few persons have succeeded in obtaining a pure culture, i.e. a culture without bacteria. Trussel (1940) obtained a bacteria-free culture of *T. vaginalis*, and later Adler and Pulvertaft (1944) and Johnson, Trussel, and Jahn (1945) succeeded in obtaining a pure culture by utilizing penicillin. Subsequently Quisno and Foter (1945) and others reported experiments in which penicillin and streptomycin were utilized. In our country, Asami (1952), by using an original culture method and adding penicillin, succeeded in making a pure culture of *T. vaginalis*.

In view of the fact that a pure culture is necessary in order to know the various biological properties of *Trichomonas*, to ascertain its pathogenicity to the human body and for the study of medicines for its treatment, we made experiments for this purpose and succeeded easily in culturing *T. vaginalis* free from bacteria as shown below.

EXPERIMENTS

1. *Culture method.* The culture tubes used by Hall (1915; 1919) for culturing anaerobic bacteria were employed. The use of these culture tubes was the most important feature of our experiments.

2. *Culture media.* We used Vf bouillon (bouillon de viande et de foie de boeuf — broth of beef meat and liver) of Weinberg and Goy (1924) to which was added 10 per cent human or horse serum and 0.5 per cent glucose. For the isolating culture we dispensed 20,000 units of crystalized penicillin into culture tubes by first dissolving 100,000 units of penicillin in 5 cc. of Vf bouillon and putting 1 cc. (20,000 units) of this solution into each culture tube.

The Vf bouillon was made according to the following method:

Beef	200 g.
Beef liver	50 g.
Hydrochloric acid C. P.	10 cc.
Pepsin	0.5 cc.
Water	1,000 cc.

We mixed the above ingredients, heated the mixture for 24 hours at 48°C. stirring it occasionally. Next we heated it for 10 minutes at 80°C., filtered it through filter paper and heated it for 25 minutes at 100°C. Correcting it to pH 5.4 with 10 per cent hydrochloric acid, we heated it for 25 minutes at 100°C., and after filtering, it was poured into Hall's culture tubes sterilized by heating for 30 minutes at 100°C. daily for 3 days and then stored. Where glucose was used, we made a 3 per cent solution, sterilizing it by heating for 15 minutes at 100°C. daily for 3 days, and added this solution to the above-mentioned Vf bouillon in

such a way as to make the glucose content 0.5 per cent. In using serum, we added the serum which had been rendered bacteria-free and had been stored beforehand to the content of the culture tubes to the extent of 10 per cent.

3. *Culture technique.* Aspirating the liquid collected in the posterior fornix of a patient with a capillary pipette, we transplanted a small amount of it in the bottom of a Hall's culture tube and kept it at 37°C. We continued to transplant

Fig. 1. Hall's Tube (1:3.5)

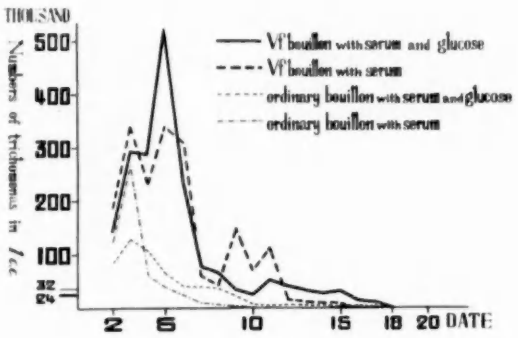

Fig. 2

on the same culture medium at intervals of 2–7 days. When it appeared under the microscope that the *T. vaginalis* culture was bacteria-free, we tested its sterility for miscellaneous bacteria by means of serum bouillon, blood agar, Vf deep gelatin and thioglycolate medium.

4. *Result.* When we made isolations in this way, the cultures of *T. vaginalis* were usually pure in the second culture or in the fifth at the latest.

When growth of *T. vaginalis* was tested in the four kinds of culture: (a) Vf bouillon plus serum and glucose, (b) Vf bouillon plus serum only, (c) ordinary

bouillon plus serum and glucose and (d) ordinary bouillon plus serum only, (a) proved to be superior both in rapidity of growth and longevity, since in about 5 days in this medium *T. vaginalis* increased to 400,000 per cc. and lived for about 18 days. Next came (b) and (c), but the growth in (d) was extremely meager. The result is shown in Figure 2.

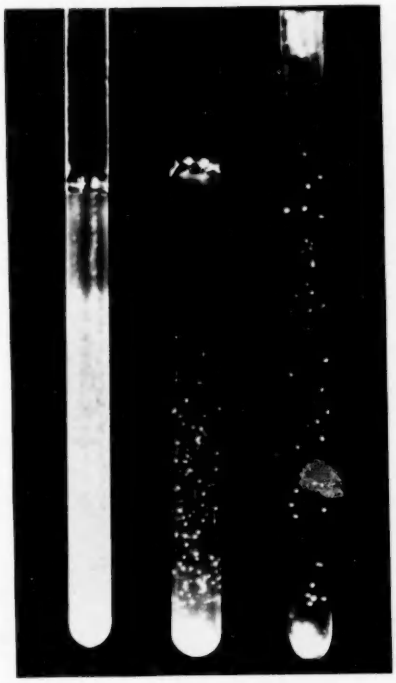

FIG. 3. Colonies of *T. vaginalis* on the 7th day culture (4th generation)

When *T. vaginalis*, which had been cultured bacteria-free in Vf bouillon, was transplanted into Vf deep agar, it formed colonies as shown in Figure 3.

Our experiments have shown clearly that *T. vaginalis* grows anaerobically.

CONCLUSION

By utilizing Vf bouillon with penicillin and Hall's tubes we have easily succeeded in culturing *T. vaginalis* free from bacteria.

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TERRAMYCIN TREATMENT OF BALANTIDIASIS IN HONDURAS

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The favorable effect of antibiotics in the therapy of human balantidiasis has recently been demonstrated by workers in Chile and in Cuba on small groups of cases.

At the University of Chile, Neghme and his coworkers found that both aureomycin and terramycin obtained a satisfactory *in vitro* response against *Balantidium coli* (Agosin *et al.*, 1950a, 1950b; Neghme *et al.*, 1952). Their published clinical trials (Neghme *et al.*, 1951) are limited to four patients who received aureomycin and one who received terramycin; all five of these patients responded well to the antibiotic treatment.

TABLE 1

The treatment of balantidium infestation with terramycin

CASE NO.	AGE	SEX	PREVIOUS UNSUCCESSFUL TREATMENT	DAILY DOSE OF TERRAMYCIN	LENGTH OF TREATMENT	SIDE EFFECTS	LENGTH OF FOLLOW-UP	NO. OF POST-TREATMENT STOOLS	RESULTS
1.	14	M	Carbarsone	1.5 Gr	10 days	None	5 months	10	Cure
2.	22	M	—	2.0 Gr	10 days	None	5 months	8	Cure
3.	6	F	—	0.75 Gr	10 days	Vomited twice	5 months	14	Cure
4.	30	M	—	2.0 Gr	10 days	None	5 months	6	Cure
5.	22	F	S T B.	2.0 Gr	10 days	None	4 months	7	Cure
6.	12	M	—	1.5 Gr	10 days	None	4 months	8	Relapse at 3 months
7.	18	M	—	2.0 Gr	10 days	Occasional nausea	4 months	11	Cure
8.	40	M	—	2.0 Gr	10 days	None	4 months	9	Cure
9.	8	F	Carbarsone	1.0 Gr	10 days	None	4 months	8	Cure
10.	16	M	—	2.0 Gr	10 days	None	3 months	6	Cure

In Cuba, Castellanos and his associates (1950) were favorably impressed with bacitracin. Also in Cuba, Burrows and Jannes (1952) treated six patients with aureomycin; in all of these, *B. coli* disappeared from the stools within four days and remained absent during a follow-up period of two to twelve weeks' duration.

Two of these recent papers (Neghme *et al.*, 1952; Burrows and Jannes, 1952) have reviewed the status of other types of compounds as described in the literature and this information need not be repeated in this presentation.

MATERIAL AND METHODS

The present study was conducted near the north coast of Honduras in the Tela Railroad Company (United Fruit Company) hospital at La Lima. Ten patients

with *B. coli* were treated between April and July 1952, with the doses of terramycin indicated in Table 1. The ages ranged from 6 years to 40 years. Seven patients were males and three were females. All came from banana labor camps where they were in close and frequent contact with pigs. Three had had previous unsuccessful treatment with other drugs; the other seven had received no medication.

RESULTS

In none of the ten patients was there a serious toxic reaction to terramycin. A six year old girl vomited twice and an 18 year old boy complained of occasional nausea; in neither instance was it necessary to discontinue the drug administration.

During the three to five months post-treatment follow-up period, 6 to 14 stool examinations were made in each case. All except one remained free of the parasite; *B. coli* was again present in the stool of a twelve year old boy three months after treatment. This boy, however, was in constant contact with pigs after completion of therapy and reinfection is considered a probability.

The effect of terramycin against other intestinal parasites was not tabulated.

SUMMARY AND CONCLUSIONS

Ten individuals infected with *Balantidium coli* responded very satisfactorily to terramycin. In all instances, symptoms disappeared quickly, and stools became negative within four days. There was no drug intolerance which necessitated discontinuance of therapy. One relapse (possible reinfection) was noted among the ten patients; this was in a 12 year old boy whose intimacy with pigs unfortunately continued after completion of his treatment. Terramycin obviously is an excellent therapeutic agent against human balantidiasis.

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SALMONELLOSIS IN MAN AND ANIMALS IN SOUTHWEST GEORGIA

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In a study done in south Texas, Watt and DeCapito (1950) reported on the striking similarity of the frequency and distribution of *Salmonella* types isolated from man as compared with those isolated from domestic animals. The fact that the same type of *Salmonella* organisms could be isolated with ease from both man and animals in the same locality indicated a very close interrelationship between the human and animal reservoirs of salmonellosis. The chance of spread from animal to man and from man to animal was quite apparent since chickens, dogs and cats were found to be the animals frequently infected with *Salmonella* organisms and these animals are in intimate contact with human beings. Consideration of this relationship between the human and animal reservoirs of salmonellosis would influence any control measures contemplated.

Certain local variations were noted in the results of the south Texas study which indicated that in order to clarify and establish some of the epidemiology of salmonellosis, similar studies in a different geographic area should be done. An opportunity to study the prevalence and distribution of *Salmonella* types in man and domestic animals in a different location was afforded when investigations of fly control and its effect on human disease were begun in southwest Georgia early in 1949.

METHODS

Representative areas of nine rural towns and one semirural town in Thomas County, Georgia, which had been chosen for the fly control investigations, were used in the study. Efforts were made to obtain a rectal swab culture at monthly intervals from all children under 10 years of age in these areas. As soon as an individual was found to harbor *Salmonella* organisms, all domestic animals kept in the close vicinity of this individual's home were cultured for members of the *Salmonella group*.

Rectal swabs obtained from the children were plated immediately on S-S agar[1] and then placed into tetrathionate enrichment broth. After a period of incubation, the broth was also plated on S-S agar. Suspicious colonies were picked from these plates and isolation of organisms of the *Salmonella* group was carried out. Rectal or cloacal swabs obtained from the animals were put directly into tetrathionate enrichment broth and returned to the laboratory for definitive isolation.[2]

[1] Shigella-Salmonella agar, Difco Laboratories, Detroit, Michigan.

[2] The final typing of all *Salmonella* organisms was done by Dr. P. R. Edwards, Communicable Disease Center, Public Health Service, Chamblee, Georgia.

RESULTS

From April 1949 to January 1952, 26,932 rectal swab cultures were done on children under 10 years of age and 21,753 similar cultures were done on yard animals. Table 1 shows the number and percent of successful isolations of *Salmonella* organisms from these cultures and, for comparison, the prevalence of *Salmonella* infections found in the Texas study. The relationship between the prevalence rates found in the children and yard animals in this study is similar to that found in the south Texas study although the percentage of isolations of *Salmonella* organisms was higher in the Texas area.

TABLE 1

Prevalence of Salmonella *infections in children and yard animals in southwest Georgia, April 1949–January 1952, and in south Texas, March 1946–May 1948.*

SOURCE	NO. RECTAL CULTURES	SOUTHWEST GEORGIA		SOUTH TEXAS
		No. positive *Salmonella*	Per cent positive *Salmonella*	Per cent positive *Salmonella*
Yard animals	21,753	202	0.9	1.4
Children under 10	26,932	136	0.5	0.6

TABLE 2

Prevalence of Salmonella *infection in various animal species in southwest Georgia and in south Texas*

ANIMAL SPECIES	NUMBER CULTURED	SOUTHWEST GEORGIA		SOUTH TEXAS
		Number positive	Per cent positive	Per cent positive
Chicken	20,189	149	0.7	0.8
Dog	705	36	5.1	3.4
Duck	401	11	2.7	2.5
Cat	321	5	1.6	3.0
Cow	21	1	4.8	0.6
Also cultured: (negative)	24 pigs	20 Guinea hens		7 Mules
	31 Turkeys	14 Rabbits		
	15 Goats	5 Pigeons		

A further breakdown of the number of positive cultures obtained from yard animals by species of animal cultured is given in Table 2. Prevalence rates of *Salmonella* infection found in the various domestic animals in the two areas are given for comparison. Twelve different animal species were cultured during this study. Chickens, dogs, ducks and cats were most often cultured because they were the kinds of animal kept in the rural towns in this area. Considering those animals cultured in significant numbers (chickens, dogs, cats and ducks), dogs had the highest prevalence rate, followed by ducks, cats and chickens. Such a relationship was also found in south Texas and the per cent positive was approx-

TABLE 3

The frequency and distribution of various Salmonella *types isolated from man and domestic animals in southwest Georgia*

KAUFFMAN WHITE GROUP	TYPE NAME	SOURCE		KIND OF ANIMAL
		Human	Animal	
B	*S. typhi-murium*	39	26	Dog, cow, cat, chicken, duck
	S. derby	15	15	Dog, chicken, cat
	S. paratyphi B	5	2	Chicken, duck
	S. california	4	0	
	S. bredeney	1	2	Dog
	Total	64	45	
C_1	*S. montevideo*	4	9	Dog, chicken, duck
	S. bareilly	1	0	
	S. tennessee	2	0	
	S. thampson	0	1	Dog
	S. oranienburg	0	1	Chicken
	Total	7	11	
C_2	*S. newport*	12	15	Dog, chicken
	S. muenchen	7	6	Cat, chicken
	S. litchfield	4	2	Dog
	S. manhattan	15	2	Dog, chicken
	S. tallahassee	0	1	Dog
	Total	38	26	
D	*S. miami*	13	10	Chicken, dog, cat
	S. berta	1	0	
	S. pensacola	1	0	
	S. pullorum	0	85	Chicken, duck
	Total	15	95	
E_1	*S. anatum*	9	8	Dog, chicken, duck
	S. meleagridis	0	1	Chicken
	S. give	0	4	Chicken
	Total	9	13	
E_2	*S. newington*	0	1	Chicken
F	*S. rubislaw*	1	0	
G	*S. worthington*	1	2	Chicken
H	*S. florida*	0	7	Dog, chicken
J	*S. kentucky*	1	1	Dog
Ungrouped	*S. duval*	0	1	Chicken
Total all groups		136	202	
Total types isolated 28		19	22	

imately the same. However, since chickens are many times more numerous than the other domestic animals, they assume considerable importance as a reservoir of salmonellosis.

TYPES OF SALMONELLA FOUND

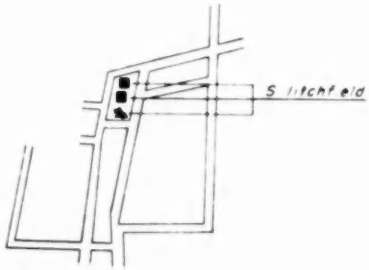
Table 3 demonstrates the frequency and distribution of the various *Salmonella* types found in man and in domestic animals during this study, and the kinds of animal found harboring each *Salmonella* type. Twenty-eight different types of *Salmonella* organisms were isolated from man and animals, 19 types were isolated from human beings and 22 types from the various domestic animals. The distribution of the 22 types isolated from domestic animals was as follows: chickens, 17 types; dogs, 13 types; ducks, 5 types; cats, 4 types; and one type from a cow. The finding of *S. pullorum* exclusively in poultry again demonstrated the host preference of this particular *Salmonella*. This limited host range is a characteristic which it shares with *S. typhi*.

A significant point of similarity between this study and the Texas study is the fact that of all the types of *Salmonella* organisms isolated, one-fourth of those isolated in each area made up 75% of the total isolations in that area, and could be divided into groups: those which were isolated in quantity in both areas, and those which were isolated frequently only in one area. For example, *S. miami* and *S. muenchen* were frequently isolated from man and animals in south Georgia but were not found in any quantity in the Texas area. Similarly, *S. javiana* and *S. oranienburg* were found in quantity in man and animals only in the Texas area. On the other hand, *S. newport*, *S. typhi-murium*, *S. derby* and a few other types were frequently isolated in man and animals in both areas. The frequent occurrence of the same *Salmonella* types in man and animals in many geographic areas suggests an animal-man relationship; the fact that some *Salmonella* organisms which are peculiar to one geographic area can be isolated in quantity from both man and animals there points even more directly to the possibility of animal-man interchange of *Salmonella* organisms.

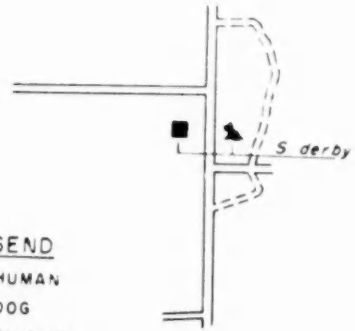
RELATION OF HUMAN POSITIVES TO ANIMAL POSITIVES

Spot maps of portions of some of the towns in the study area have been constructed to demonstrate the types of *Salmonella* organisms found in these areas in man and animals at approximately the same time, in order to show a more intimate relationship between human positives and animal positives. The maps are drawn to scale and symbols are located at the approximate location of the positives. All *Salmonella* organisms isolated during a short interval of time in a given area are depicted by the symbols on the spot maps. Human beings are represented by small squares; chickens and dogs are shown by small figure drawings. Solid lines drawn from the symbols to the name of the *Salmonella* type indicate that it was isolated from both a human being and an animal at the same time.

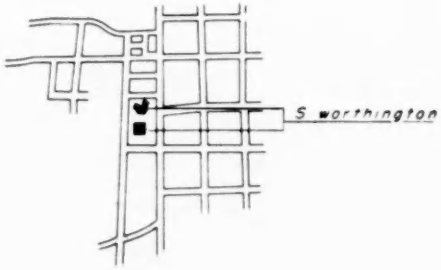
Four examples are given in Plate I in which an identical *Salmonella* type was isolated from a human being and a domestic animal in the same environment at



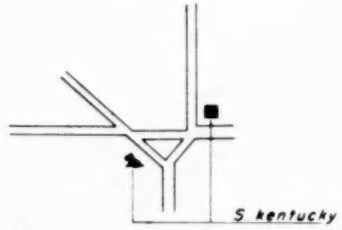
LEGEND
■ - HUMAN
🐕 - DOG



LEGEND
■ - HUMAN
🐕 - DOG
🐓 - CHICKEN



LEGEND
■ - HUMAN
🐕 - DOG
🐓 - CHICKEN



LEGEND
■ - HUMAN
🐕 - DOG

PLATE I

approximately the same time. These examples were selected because each of them shows a *Salmonella* type which was infrequently isolated during the course of this study, but when found, it was from man and from animals in close association with each other in the same environment and at the same time. *S. kentucky* was isolated only once in man and was found in a dog in the same environment at that time. *S. litchfield* was isolated four times in man and in one of these instances it was also found in an animal at that individual's home. *S. worthington* was isolated only once from man and also was found in an animal in that locale at the same time. *S. derby* was isolated frequently during the course of the study but was only isolated once in the area shown on the spot map; it was also found in an animal at that time in that area. These examples are further evidence that in a given area there is a considerable interrelationship between salmonellosis in man and salmonellosis in animals. This interrelationship, which is again demonstrated, does not provide evidence as to how the spread of infection occurs or in which direction it travels.

DISCUSSION

The findings in south Texas and these from south Georgia show that *Salmonella* organisms are easily found in man and in his domestic animals. A few *Salmonella* types make up the majority of the isolations; some are isolated in both areas and some are peculiar to one location. The types found in human beings in each geographic area were also commonly found in the domestic animals in that location.

Two conclusions are drawn from these results.

1. There is an impressive similarity between the distribution of various *Salmonella* types found in man and in domestic animals in the same environment.

2. There is endemic salmonellosis in man and in domestic animals, and an interrelationship between the two reservoirs is apparent.

The intimacy of contact between human beings and dogs, cats and chickens makes it obvious that direct spread from animals to man or from man to animals can easily take place. This phase of the epidemiology of salmonellosis will remain obscure until the actual routes of transmission have been established and their relative importance determined.

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IMPROVED MEDIA FOR *VIBRIO CHOLERAE* AND SALMONELLA USED IN HUMAN VACCINES

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The present investigation started from the observation that glycerol in the presence of phosphate was a powerful nutritive for *Vibrio cholerae* (Kaplan and Koch, 1952). *V. cholerae* is not a fastidious organism but the growth obtained is rather sparse and a large number of organisms is used in immunization. Sparse growth is a serious obstacle in diagnostic work (Felsenfeld *et al.*, 1951) and in vaccine production. It is estimated that several hundred million doses would be needed yearly all the world over.

Growth of salmonella is even poorer than that of *V. cholerae*. Fortunately, however, the doses of salmonella used in vaccination against typhoid and paratyphoid are smaller than those of *V. cholerae* employed in vaccination against cholera; on the other hand large populations are continuously being vaccinated against diseases caused by salmonella. Thus even a slight improvement of the known media means an appreciable saving in materials and labor in both instances.

Salmonella intended for vaccines are mostly grown on solid media; *V. cholerae* is grown on solid as well as in fluid media. The media to be described were all solid and contained 2.5% agar; they had a pH of 7.6–7.8 when prepared for *V. cholerae*, and a pH of 7.4–7.6 for salmonella. Five ml. of the medium were dispensed into 50 ml. flat bottom flasks which proved to be better than slants, for the inoculum could be spread more evenly and the organisms were easily harvested. Furthermore, an estimate of yield per square centimeter of the small flask allowed one to predict fairly well the yield obtainable from the big bottle used in vaccine production. In the small flasks 5 ml. of agar had a free surface of about 18 square centimeters. After an incubation of 22–24 hours the growth was harvested by rinsing with 20 ml. of saline containing 0.01 per cent merthiolate. The suspension was then heated in a waterbath at 56°C. for half an hour, and the growth estimated turbidimetrically (Koch and Kaplan, in press). The bacterial number obtained, times 20 (for 20 ml. saline were used for rinsing), and divided by 18 (the free surface of the agar in the flask was 18 cm^2), indicated the yield per square centimeter of agar. To minimize errors when estimating the comparative yield of various media, the harvest from every experimental medium was always compared with that from a reference standard medium, sown and incubated simultaneously. Thus the gain due to improved composition of an experimental medium could also be expressed as the relationship:

$$\frac{\text{Harvest from experimental medium}}{\text{Harvest from reference standard medium}}$$

This fraction was called Multiplication Factor; multiplication factors are shown, in addition to yields per square centimeter of agar surface, in Table 7. The choice

of a reference standard medium obviously was arbitrary. For *V. cholerae* a recently advocated salt-peptone medium (Felsenfeld *et al.*, 1951) was adopted as standard, while for salmonella, Bacto Nutrient Agar was chosen for comparison. Triplicates were always taken and in the case of any suspected improvement the medium was twice prepared anew; the experiments were then repeated in quadruplicate.

VIBRIO CHOLERAE

(Strains investigated: No. 41–1 ((Ogawa)) and No. 35A3 ((Inaba))[1])

Glycerol in the presence of phosphate was found to be a powerful nutritive for *V. cholerae* (Kaplan *et al.*, 1952; Koch *et al.*, in press). Both strains showed a very similar growth-response when glycerol (2.2%) and disodium-hydrogen-phosphate (0.25%) were incorporated into the media. The steps which led to the medium shown below will be published elsewhere (Koch *et al.*, in press). 100 ml. of this cholera medium contained (in grams): Peptone (Difco) 0.5, Casein hydrolysate (Squibb) 0.5, NaCl 0.5, Na_2HPO_4 0.25, Bovril 0.15, Marmite 0.15, Glycerol 2.2, and Agar 2.5; its pH was 7.6–7.8. The yield from this medium was about 12.6 times that of the salt-peptone medium mentioned above, containing 1.0 g Difco Peptone, 0.5 g. NaCl and 2.5 g. Agar per 100 ml. With an inoculum of approximately 1.3×10^6 organisms per square centimeter of agar surface, the harvest from the improved medium was 13.2×10^9 organisms per square centimeter, while the standard medium containing salt and peptone yielded only 1.05×10^9 organisms. *V. cholerae*, grown on this medium, possessed an antigenic power satisfying the requirements of the U. S. Public Health Service.

SALMONELLA

(Strains investigated: *Salmonella typhi* Ty2, 779 and T63[2]; *Salmonella paratyphi A*, *Salmonella paratyphi B* and *Salmonella paratyphi C*.)

In view of their importance to highly developed countries, salmonella have always attracted much more attention than *V. cholerae*; thus there already exist a number of media which are fully described in Wadsworth's Standard Methods (1947). In recent attempts to simplify and cheapen salmonella media for diagnostic purposes, Messerschmidt and Pöhlig (1950) and Messerschmidt (1952) employed tryptic digests of skimmed milk as well as peptic and tryptic digests of meat.

In the present investigation, 48 salmonella media were investigated. They differed in their main source of nitrogen which was peptone, casein hydrolysate, or a mixture of both. Two brands of peptone were used (Armour and Difco), and one brand of casein hydrolysate (Squibb). Other variations in the composition were due to the presence or absence of phosphate, yeast extract (Marmite), meat extract (Bovril), glycerol and glycerophosphate. All media were sown with 500,000 organisms per square centimeter of agar. The composition of the media

[1] We are much indebted to Professor L. E. Ranta for both strains.

[2] *S. Typhi* Ty2 is mainly used in Great Britain, strain 779 and T63 in the U. S.

as well as their yield is shown in the accompanying tables. Tables 1–6 list in each row the number of the medium, its components (in grams per 100 ml.) and the yield per square centimeter after 22–24 hours incubation. Table 7 also shows Multiplication Factors, taking the harvest from a Difco nutrient agar medium as unity. As the three typhoid strains tested responded on all media similarly, their responses were pooled in all tables.

Plain media containing nothing but a nitrogen source, salt and agar are shown in Table 1. The yields of media Nos. 1, 23, and 32 were practically unchanged by the addition of phosphate (Nos. 3, 25, and 35).

TABLE 1

The effect of phosphate on plain media

NO. OF MEDIUM	PEPTONE (DIFCO)	CASEIN HYDROLYS. (SQUIBB)	NaCl	Na_2HPO_4	AGAR	ORGANISMS × 10^9/cm^2			
						Salm. Typhi	Para "A"	Para "B"	Para "C"
1	1.0		0.5		2.5	0.81	0.83	0.77	0.74
3	1.0		0.5	0.25	2.5	0.61	0.66	0.73	0.54
23	0.5	0.5	0.5		2.5	0.75			
25	0.5	0.5	0.5	0.25	2.5	0.74			
32		1.0	0.5		2.5	0.47	0.30	0.53	0.43
35		1.0	0.5	0.25	2.5	0.47	0.41	0.47	0.42

TABLE 2

The effect of meat-extract and yeast-extract on plain media

NO. OF MEDIUM	PEPTONE (DIFCO)	CASEIN HYDROLYS. (SQUIBB)	NaCl	BOVRIL	MARMITE	AGAR	ORGANISMS × 10^9/cm^2			
							Salm. Typhi	Para "A"	Para "B"	Para "C"
1	1.0		0.5			2.5	0.81	0.83	0.77	0.74
5	1.0		0.5	0.15	0.15	2.5	1.02	0.93	0.93	1.02
32		1.0	0.5			2.5	0.47	0.30	0.53	0.43
38		1.0	0.5	0.15	0.15	2.5	0.79	0.62	0.83	0.96

Supplementing, however, the same plain media (Nos. 1 and 32) with Bovril and Marmite instead of phosphate (Nos. 5 and 38), the yield was in both instances increased. (see Table 2).

A comparison between medium No. 5 and No. 38 in Table 2 also suggests the superiority of peptone over casein hydrolysate as a source of nitrogen.

While Table 1 shows the action of phosphate, and Table 2 that of Bovril and Marmite, Table 3 demonstrates the joint action of all three of them, namely phosphate, Bovril and Marmite.

The plain salt-peptone medium, already shown in Tables 1 and 2, gained definitely from this joint action (compare No. 1 with No. 7 in Table 3). Medium No. 18 was a variant of medium No. 7, obtained by taking peptone Armour instead of peptone Difco. Medium No. 29 differed from No. 7 only in its source of nitro-

gen which was half peptone and half casein hydrolysate in medium No. 29. A comparison of the three supplemented media, namely Nos. 7, 18 and 29 indicates that Difco peptone (No. 7) was better than the peptone-casein hydrolysate medium (No. 29), though Armour peptone (No. 18) gave still higher yields.

Burrows (1942) observed that most strains of *S. typhi* were able to utilize glycerol in a synthetic medium containing various amino-acids; he also noted that the amino-acids were better utilized in the presence of glycerol. More recently, Davis and his associates (1950, 1952), were able to show that *S. oranienburg* and *S. anatum* but not *S. pullorum* utilized glycerol in a synthetic medium.

TABLE 3

The joint effect of phosphate, meat-extract and yeast-extract on plain media

NO. OF MEDIUM	PEPTONE (DIFCO)	PEPTONUM SICCUM (ARMOUR)	CASEIN HYDROLYS. (SQUIBB)	NaCl	$NaHPO_4$	BOVRIL	MARMITE	AGAR	ORGANISMS × 10^9/cm²			
									Salm. Typhi	Para "A"	Para "B"	Para "C"
1	1.0			0.5				2.5	0.81	0.83	0.77	0.74
7	1.0			0.5	0.25	0.15	0.15	2.5	1.02	0.87	1.12	0.97
18		1.0		0.5	0.25	0.15	0.15	2.5	1.19	1.19	1.55	1.43
23	0.5		0.5	0.5				2.5	0.75			
29	0.5		0.5	0.5	0.25	0.15	0.15	2.5	0.90	0.88	1.05	0.92

TABLE 4

The effect of glycerol on plain media

NO. OF MEDIUM	PEPTONE (DIFCO)	CASEIN HYDROLYS. (SQUIBB)	NaCl	GLYCEROL	AGAR	ORGANISMS × 10^9/cm²			
						Salm. Typhi	Para "A"	Para "B"	Para "C"
1	1.0		0.5		2.5	0.81	0.83	0.77	0.74
2	1.0		0.5	2.2	2.5	1.76	1.65	1.70	0.67
32		1.0	0.5		2.5	0.47	0.30	0.53	0.43
34		1.0	0.5	2.2	2.5	1.65	1.80	2.40	1.02

All three typhoid strains tested grew far better in the presence of glycerol, and in repeated titrations it was found that the optimal concentration was 2.2% glycerol, incidentally the same amount as for *V. cholerae*. Table 4 shows this effect of glycerol in a simple peptone medium (No. 1) and a casein hydrolysate medium (No. 32) respectively (No. 2 & No. 34). With peptone (compare No. 1 with No. 2) the yield of *S. typhi*, *S. paratyphi A* and *S. paratyphi B* was doubled by the addition of glycerol; only the yield of *S. paratyphi C* remained unchanged. A casein hydrolysate medium (No. 32 & 34) gained even more from the addition of glycerol than the peptone medium, and, in contrast with the peptone medium, the harvest of *S. paratyphi C* was increased as well.

In the presence of Bovril and Marmite, a salt-peptone medium (No. 16 in Table 5) could be very much improved by incorporating glycerol (No. 17) even in the absence of phosphate. Here again the exception proved to be *S. paratyphi*

C where phosphate as well as glycerol was needed in order to raise the yield from 1.43×10^9 organisms/cm² to 2.59×10^9 organisms/cm². (No. 19). Medium No. 19 not only raised appreciably the harvest of *S. paratyphi C*, it also was found to suit *S. typhi*, *S. paratyphi A* and *S. paratyphi B* as well, as can be seen from Table 5. The same table also shows media analogous to medium No. 19, but with the peptone partly (No. 30) or entirely (No. 41) replaced by casein hydrolysate. Medium No. 30 was a trace better than medium No. 19 for growing *S. paratyphi A* and *S. paratyphi C*, but it was definitely inferior for *S. typhi* as well as for *S. paratyphi B*. The parallel casein hydrolysate medium (No. 41) was worse than medium No. 19 for all salmonellas tested.

On Medium No. 19 *S. typhi* and *S. paratyphi B* showed a very high yield, while the harvest of the other two strains, though much improved, was noticeably less; consequently another approach was tried.

TABLE 5

Plain media supplemented with phosphate, meat-extract, yeast-extract and glycerol

NO. OF MEDIUM	PEPTONE (DIFCO)	PEPTONUM SICCUM (ARMOUR)	CASEIN HYDROLYS. (SQUIBB)	NaCl	$NaHPO_4$	BOVRIL	MARMITE	GLYCEROL	AGAR	ORGANISMS $\times 10^9$/cm²			
										Salm. Typhi	Para "A"	Para "B"	Para "C"
16		1.0		0.5		0.15	0.15		2.5	1.20	1.07	1.35	1.43
18		1.0		0.5	0.25	0.15	0.15		2.5	1.19	1.19	1.55	1.43
17		1.0		0.5		0.15	0.15	2.2	2.5	3.25	2.20	3.40	1.43
19		1.0		0.5	0.25	0.15	0.15	2.2	2.5	4.40	3.08	4.60	2.59
29	0.5		0.5	0.5	0.25	0.15	0.15		2.5	0.90	0.88	1.05	0.92
30	0.5		0.5	0.5	0.25	0.15	0.15	2.2	2.5	3.41	3.50	3.60	2.69
40			1.0	0.5	0.25	0.15	0.15		2.5	0.84	0.83	1.15	0.90
41			1.0	0.5	0.25	0.15	0.15	2.2	2.5	3.24	2.20	3.45	2.20

We found that salts of glycero-phosphoric acid were utilized by salmonella. The sodium and the calcium salts of glycero-phosphoric acid were tried. Calcium-glycero-phosphate, though promoting growth, was not very suitable as it is only slightly soluble in cold water (2 g./100 ml.) and even less in hot water; furthermore, when collecting the harvest by rinsing, the growth came off in flakes and had to be broken up by shaking. Sodium-glycero-phosphate could be easily incorporated into media as it is very soluble in water.

Varying amounts of sodium-glycero-phosphate were added to media containing a source of nitrogen, salt, Bovril and Marmite. The optimal concentration was found to be 3.0 g./100 ml. medium but the harvest was further improved by the incorporation of 0.7 g. glycerol per 100 ml. of medium.

Table 6 shows three sodium-glycero-phosphate media which differed in their glycerol content. Medium No. 20 did not contain glycerol, while media Nos. 21 and 22 contained 0.7 g./100 ml. and 1.4 g./100 ml. respectively; medium No. 21 was the best among the three media in Table 6.

The reference standard medium, Difco nutrient agar (No. 42 in Table 7), gave a rather low yield, ranging between 0.46×10^9 and 0.78×10^9 organisms

per square centimeter of agar for the strains tested. Though the reference standard medium also gained from the addition of glycerol (see medium No. 43 in Table 7), this did not apply to *S. paratyphi C*. Here again (medium No. 44) phosphate was needed in addition to glycerol. The reference standard medium, even when supplemented with phosphate and glycerol (medium No. 44) was only equal to medium No. 18 which did not contain glycerol.

TABLE 6

The effect of glycerol and sodium-glycero-phosphate

NO. OF MEDIUM	PEPTONUM SICCUM (ARMOUR)	NaCl	BOVRIL	MARMITE	GLYCEROL	SODIUM-GLYCERO-PHOSPHATE	AGAR	ORGANISMS × 10^9/cm²			
								Salm. Typhi	Para "A"	Para "B"	Para "C"
20	1.0	0.5	0.15	0.15		3.0	2.5	2.95	3.00	3.60	2.92
21	1.0	0.5	0.15	0.15	0.7	3.0	2.5	4.07	3.40	4.00	4.04
22	1.0	0.5	0.15	0.15	1.4	3.0	2.5	3.63	3.60	3.90	3.41

TABLE 7

Comparison of the Reference Standard Medium (Difco Bacto nutrient agar) with those proposed

NO. OF MEDIUM	PEPTONUM SICCUM (ARMOUR)	NUTRIENT AGAR* (DIFCO)	NaCl	$NaHPO_4$	BOVRIL	MARMITE	GLYCEROL	SODIUM-GLYCERO-PHOSPHATE	AGAR	ORGANISMS × 10^9/cm²				MULTIPLICATION FACTOR, YIELD FROM MEDIUM NO. 42 TAKEN AS UNITY			
										Salm. Typhi	Para "A"	Para "B"	Para "C"	Salm. Typhi	Para "A"	Para "B"	Para "C"
42		3.1*								0.78	0.46	0.75	0.65	1.00	1.00	1.00	1.00
43		3.1*					2.2			1.25	0.82	1.50	0.65	1.60	1.78	2.00	1.00
44		3.1*		0.25			2.2			1.32	0.88	1.60	1.32	1.69	1.91	2.13	2.03
18	1.0		0.5	0.25	0.15	0.15			2.5	1.19	1.19	1.55	1.43	1.53	2.59	2.07	2.20
19	1.0		0.5	0.25	0.15	0.15	2.2		2.5	4.40	3.08	4.60	2.59	5.64	6.70	6.13	3.98
21	1.0		0.5		0.15	0.15	0.7	3.0	2.5	4.07	3.40	4.00	4.04	5.22	7.40	5.33	6.22

* 3.1 g nutrient agar contain:

Bacto-beef extract	0.3 g
Bacto-peptone	0.5 g
Sodium chloride	0.8 g
Bacto-agar	1.5 g

While the multiplication factors for the supplemented reference standard medium (No. 44) ranged between 1.69 and 2.13, the corresponding range for our medium No. 19 was between 3.98 and 6.70 (see Table 7). Comparing medium No. 19 with one containing sodium-glycero-phosphate (No. 21) as well, a striking improvement will be noticed for *S. paratyphi C*. Medium No. 21 gave multiplication factors between 5.22 and 7.40.

A comparison of the yields per square centimeter of agar indicates that *S. typhi* and *S. paratyphi B* should be grown on medium No. 19, while medium No. 21 is more suitable for *S. paratyphi A* and *S. paratyphi C*.

SUMMARY

A cholera medium has been developed which, mainly due to the joint action of glycerol and phosphate, gives a twelve times higher yield than a simple salt-peptone medium. This medium suits two strains, Ogawa and Inaba, commonly used in vaccine production. Two salmonella media have been designed which give good growth of the following strains employed in vaccines: *S. typhi* Ty2, 779 and T63; *S. paratyphi A*, *S. paratyphi B* and *S. paratyphi C*. The yield of these two salmonella media is between 5.6 to 7.4 times higher than that of a Bacto nutrient agar medium. This improvement is mainly due to the incorporation of glycerol, phosphate and sodium-glycero-phosphate.

ACKNOWLEDGMENT

Through the media described are easy to prepare, we thought it might perhaps be an advantage to have them in the dry state as well. Dr. H. W. Schoenlein and Dr. C. W. Christensen of Difco Laboratories very kindly agreed to prepare for us a few experimental batches of the cholera medium. The reconstituted medium, after the addition of glycerol, showed the same growth as that prepared in our laboratory.

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STUDIES ON INTESTINAL PARASITIC INFECTIONS IN CHOLBURI PROVINCE (THAILAND)[1]

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I. INTRODUCTION

The development of a comprehensive program for the control of intestinal parasitic infections in Thailand necessitates a detailed knowledge of the extent of the problem. No recent information is available on the extent of intestinal parasitic infections in Thailand and of the role that they play in affecting the health of the people. To obviate this lack of information it was decided to carry out investigations in as many provinces of Thailand as possible. The work to be reported here applies only to the first province examined, Cholburi (see Map). Similar investigations are at present being carried out in other provinces.

The purposes of this study were the following: a) The determination of the extent and severity of intestinal parasitic infections in the Cholburi Province. b) The collection of other health data and the correlation of parasitic infections with the general health status.

II. DESCRIPTION OF THE LOCALITY

Cholburi is situated on the east side of the Gulf of Thailand. The city is about 65 miles from Bangkok and has a population of approximately 20,000 people. The province is located in an area with a narrow strip of sandy soil about 2 miles wide along 80 miles of sea coast. Hilly areas adjoin this strip separating it from the plains beyond the hills. It consists of 8 districts which in turn are divided into 68 communes and 3 municipalities. The province has an area of 1747 square miles and a population of about 230,000. The soil is fertile for the most part of the Province. Along the sandy soil sugar cane, tapioca, coconut and pineapple are grown. Lumbering is the main source of income in the hilly areas. Rice cultivation in paddy fields takes place beyond the hills. Fishing is also a major occupation. The province is provided with roads reaching every district and most of the large villages. Except for some days during the rainy season all these roads are passable by jeep or by oxcart. There is no railroad passing through Cholburi Province and no civilian airfields available so that all the interprovincial communication depends on buses or fishing vessels. The public education is somewhat

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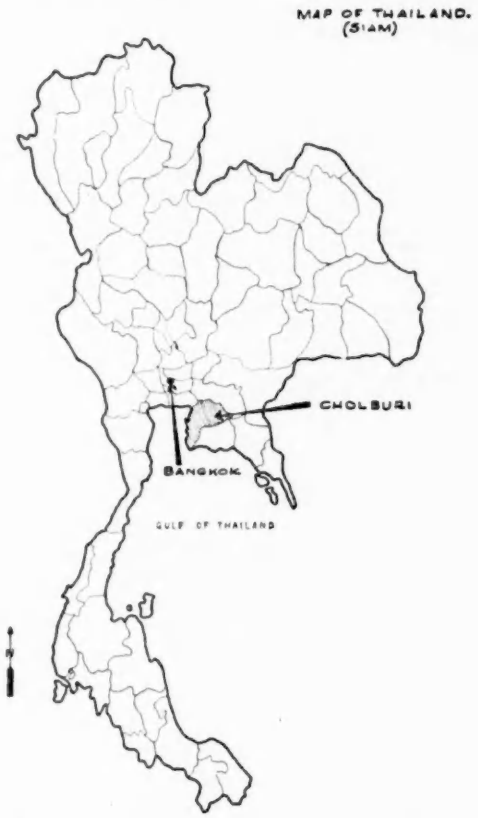
higher than that of the country as a whole. There are elementary schools and Buddhist monasteries in nearly every village. The population is almost exclusively Buddhist in religion and Thai in nationality.

Water supply, sewage and refuse disposal are poorly organized and are operated on an individual basis. Some pit latrines are available but seldom used. Rainwater is the only source of drinking water on the strip of land along the shore.



The supply from dug wells is usually unfit for use throughout the Province but is occasionally utilized.

Although there is no essential difference in the sanitation of the people living within the city limits of Cholburi (urban area) and those living in the villages (rural areas), the environmental conditions which are responsible for the survival and transmission of helminthic parasites are quite contrasting. The people of the city of Cholburi for the most part live under crowded conditions. Their activities are primarily centered around the houses where the dissemination of

Ascaris eggs is possible on a large scale. On the other hand, in the villages people live sparsely and often walk considerable distances to go to work in the fields.

TABLE 1

Summary of climatological data in Cholburi during 1951

MONTH	MEAN AIR TEMPERATURE IN C°			RAINFALL IN MM.	MEAN RELATIVE HUMIDITY PER CENT
	Max.	Min.	$\frac{\text{Max} - \text{Min}}{2}$		
January	31.4	20.2	25.8	4.5	70.8
February	32.1	21.8	27.0	9.7	75.2
March	33.5	24.5	29.0	24.0	75.0
April	34.8	26.1	30.5	32.9	73.3
May	33.3	25.2	29.3	159.4	80.4
June	32.1	25.5	28.8	106.8	80.3
July	31.9	24.5	28.2	431.3	81.3
August	32.1	25.5	28.8	152.4	78.5
September	32.0	24.5	28.3	295.6	84.7
October	31.5	24.1	27.8	211.8	82.7
November	31.4	23.2	27.3	238.5	83.3
December	30.2	20.0	25.1	7.0	73.0

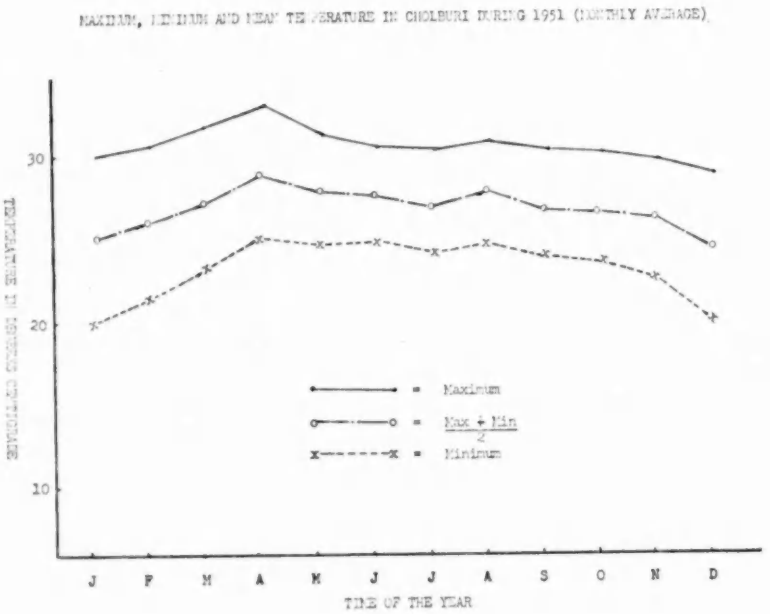

MAXIMUM, MINIMUM AND MEAN TEMPERATURE IN CHOLBURI DURING 1951 (MONTHLY AVERAGE)
TEMPERATURE IN DEGREES CENTIGRADE
30
20
10
= Maximum
= Max + Min / 2
= Minimum
J F M A M J J A S O N D
TIME OF THE YEAR

FIG. 1

There, following soil pollution, heavy rainfall and abundant vegetation make soil infection with hookworm larvae possible over extended areas.

The climate is tropical and humid. As shown in Table 1, and Figure 1, the average temperature throughout the year during which this study was carried out was between 25.1° and 30.5°C. which is the optimum for the development of the free stages of the hookworm life cycle. The minimum mean temperature was 20.0°C. in December and the maximum 34.8° in April. Both of these extremes are compatible with the survival of hookworm eggs and larvae. The annual rainfall amounting to over 1,600 mm. is also favorable for the propagation of hookworm infection. As indicated in Figure 2, there are usually two peaks of the heaviest rainfall during the year, one in July and one in September. The

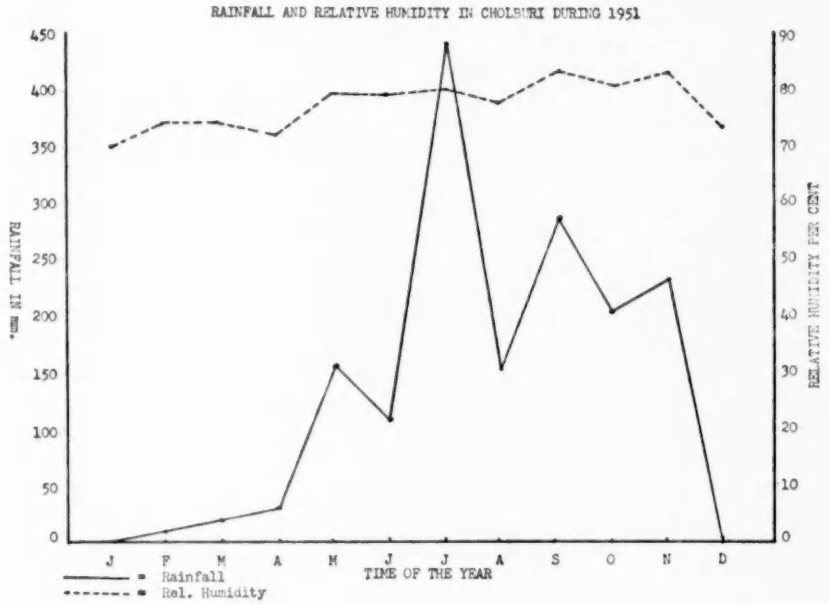


FIG. 2

statistics for the mean relative humidity indicate that it is constantly high throughout the year with little variation from month to month. As indicated in Table 1 and Figure 2, the lowest degree of relative humidity (70.8%) was in January and the highest in September (84.7%).

III. MATERIALS AND METHODS

a) *Selection of the Sample.* A total of 666 individuals is included in this study. Of these, 447 were selected within the city limits, and 219 in small villages in the Province.

In the city of Cholburi one school with a student body of 400 was selected because the children lived in different parts of the city and because the economic status of their families seemed to be conveniently placed somewhat between

the two extremes. Also, this school was the only one with a sufficient number of children of both sexes, all of them being between 5 and 14 years of age. Complete data were obtained from 387 out of the 400 children. A total of 64 Provincial officers working in the provincial building of Cholburi was also selected for this study. Complete data were obtained from 60 out of 64 officers selected.

In the rural areas it was attempted to examine as large a number of the population of each small aggregation of houses as practicable. This was possible only through the active participation of the headman of the village. Although it is not possible to state with certainty the per cent of population of each village examined because of the lack of a precise census, it is believed that a very large proportion of the people over 1 year of age residing in the area during the period of our survey was examined.

b) *Stool examinations.* Three places were selected as stations for the examination of fecal specimens. The stools were examined by the direct smear method, at least two smears being examined for each patient, one unstained and one stained with iodine. Only stools that were passed within 8 hours from the time of examination were included in this study. Those that were positive for hookworm were examined again by the Stoll dilution egg-counting technique as given by Cort, Grant and Stoll (1926). All egg counts were reduced to the basis of formed feces and in children up to 4 years they were further reduced to 50 per cent because of the small size. This was not done in children 5 to 14 years old because they show average amount of feces not statistically distinct from the adults (Cort *et al.*, 1929).

c) *Blood examinations.* Blood samples were obtained from all the urban patients for hemoglobin readings, for differential blood counts and for examination of blood parasites that may have influenced the blood picture as well as the physical findings. No patients were found to harbor hemoflagellates or microfilariae, and malaria parasites were found only in one patient (gametocytes of *Plasmodium vivax*). This case was discarded for the purposes of our study. For hemoglobin readings the Talquist scale was used. Blood smears for the differentiation of leucocytes and for the search for parasites were stained with Giemsa stain. One hundred leucocytes were differentiated as follows: monocytes, lymphocytes, basophiles, eosinophiles and neutrophiles. All stool and blood examinations were done by the authors to insure uniformity of interpretation.

d) *Physical examinations.* A physical examination was made on each urban individual. Height, weight, blood pressure, temperature and respiration were all taken by one nurse using the same instruments throughout the experiment to insure uniformity. The blood pressure, the temperature and the respiration rate were measured with the patient in the sitting position. The patients were required to rest at least 10 minutes before examination began in order to minimize the effect of physical exertion and/or excitement upon the results.

e) *Recording of data.* All the information was recorded on a punch card designed by the senior author for the purpose. The age, sex, race, religion, locality, school, grade, date and time at which the stools were passed were filled out by the teachers in the school, by the chief clerk in the Provincial Office and by the headman of

the village in the rural areas. The other information such as appearance and consistency of the stool, time interval between defecation and examination, method of examination, hemoglobin values, parasitic findings, etc., were filled out by the authors at the time of examination.

Although a helminthological survey was done in the rural as well as in the urban parts of the province, correlation studies with the general health status were possible only in the urban area where malaria is practically nonexistent.

IV. INCIDENCE OF PARASITISM IN THE CURRENT SURVEY

a) *Urban area.* Data on 387 children (212 males and 175 females) and 60 adults (42 males and 18 females) are included in this survey.

TABLE 2

The incidence of intestinal parasitic infections in the urban area of Cholburi province

PARASITES	SCHOOL CHILDREN BY AGE GROUPS								TOTAL FOR SCHOOL CHILDREN		ADULT PROVINCIAL OFFICERS	
	5–6 years		7–8 years		9–10 years		11–14 years					
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
A. lumbricoides	26	62	72	56	46	40	39	38	183	48	16	27
Hookworm	7	17	22	17	35	31	32	31	96	25	2	3
T. trichiura	5	12	17	13	11	10	14	14	47	12	2	3
E. histolytica	2	5	4	3	6	5	3	3	15	4	4	7
E. coli	7	17	12	11	16	14	15	15	50	13	4	7
E. nana	9	21	21	16	18	16	16	16	65	17	12	20
I. bütschlii	3	7	2	2	2	2	1	1	8	2	3	5
G. lamblia	5	12	26	20	27	24	21	21	79	24	13	22
C. mesnili	3	7	7	5	3	2	5	5	18	5	2	3
T. hominis	1	2	4	3	3	2	1	1	9	2	1	2
Total examined	42	—	130	—	114	—	101	—	387	—	60	—
Total infected	42	100	110	84	102	89	85	84	339	88	47	78

The results have been summarized in Table 2. The incidence of intestinal parasitism varied from 100 per cent among school children 5 to 6 years old, to 78 per cent among adult Provincial Officers. These figures are probably lower than the actual incidence. Since we employed the smear method alone, it is most likely that several very lightly infected cases were missed.

Ascaris lumbricoides was the dominant pathogenic parasite varying in its incidence from 62 per cent among children 5 to 6 years old, to 27 per cent among adult Provincial Officers. Hookworm was found in 31 per cent of children 9 to 14 years old, and only in 3 per cent of the adult officers. These findings can be, partly at least, explained by the observation that all the Provincial Officers examined work in the Provincial Building and wear shoes at least during working hours, while almost 100 per cent of the children are barefooted all the time. Other

factors such as a better diet and the development of resistance may also play a role.

Trichuris trichiura was much less common, its incidence varying from 14 to 3 per cent in the various groups. *Strongyloides* was encountered in only three children. *Taenia* and *Enterobius* were sometimes seen in the fecal smears but they have been omitted from this report since no special techniques for their detection were employed and the cases encountered would thus be misleading representing only a small fraction of the actual incidence.

The incidence of *Endamoeba histolytica* varied from 3 to 7 per cent in the various groups. It is rather surprising that the overall incidence was not higher since the water supply is very unsatisfactory and there is considerable food contamination by people and by flies which are present in great numbers. These findings, however, are in agreement with those reported for South Korea (Hunter *et al.*, 1951).

TABLE 3

The incidence of intestinal helminthic infections in the rural areas of Cholburi province

AGE GROUPS	NUMBER EXAMINED	INFECTED		HOOKWORM		ASCARIS		TRICHURIS	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
1–4	33	18	55	17	51	6	18	0	0
5–9	32	30	94	30	94	3	9	1	3
10–14	33	31	94	31	94	1	3	1	3
15–29	39	32	82	32	82	1	3	1	3
30 +	82	73	89	71	87	6	7	9	11
Total	219	184	85	181	83	17	8	14	6

Endamoeba coli, *Endolimax nana and Giardia lamblia* were the three most common protozoa. The incidence of *E. coli* varied from 7 per cent among adult Provincial Officers to 17 per cent among children 5 to 6 years old; that of *E. nana* was around 20 per cent in all age groups, and that of *G. lamblia* varied from 12 to 24 per cent in different groups.

Infections with the most common pathogenic parasites, *Ascaris*, hookworm, *Trichuris* and *E. histolytica*, were respectively: among school children—48, 25, 12, and 4 per cent; among adult Provincial Officers—27, 3, 3 and 7 per cent.

Although the sample is too small to afford detailed comparisons of the incidence in different age groups, the results in general seemed to indicate that the incidence with *A. lumbricoides* decreases with increase in age within the groups studied. This observation is in agreement with similar observations reported in similar studies from other parts of the world (Cort *et al.*, 1929). As shown in Table 2, such a clear-cut relationship of incidence of infection with age was not encountered with the other parasites.

b) *Rural areas.* The results of the stool examinations on 219 individuals from rural areas of Cholburi Province are shown in Table 3. The incidence of intestinal

helminthic infections varied from 55 per cent among children 1 to 4 years old to 94 per cent among children 5 to 14 years old, with an average for all the age groups of 85 per cent. Hookworm was the dominant pathogenic parasite varying in its incidence from 51 to 94 per cent with an overall average of 83 per cent. *Ascaris lumbricoides* was found in 18 per cent of the children 1 to 14 years old, and only in 3 per cent of the children 5 to 14 years old. *Trichuris trichiura* was found in 6 per cent of the rural population examined.

The results in general indicate that among children the incidence of hookworm increased with increase in age. Conversely, the incidence of *Ascaris* decreased with increase in age. Considering that hookworm is primarily an infection acquired in the fields and *Ascaris* is primarily an infection acquired around the house, these results can be easily interpreted.

Comparing the incidence of helminthic infections in this rural group with that of the urban group, it can be stated in general that while the incidence of *Ascaris* is higher in the city, that of hookworm is much higher in the villages. This again can be interpreted on the basis of the different epidemiology and life cycle of the two parasites.

V. QUANTITATIVE DETERMINATIONS OF HOOKWORM INFECTION

a) *Urban area.* Egg counts were done on 96 children found to be infected with hookworm. As indicated in Table 4, 55.2 per cent harbored less than 1,000 eggs per ml. of feces (EPG), and 7.3 per cent more than 10,000. The mean hemoglobin level was of 68 per cent among the children with milder infections, and of 63 per cent in those harboring 10,000 or more EPG.

Other factors which might influence the hemoglobin level of the people in the area surveyed were investigated. Malaria was present in only one case which was discarded from our series. Schistosomiasis is absent in the area studied. The nutritional level of the people in general was found to be comparatively good, a fact probably related to the frequent consumption of fish in addition to rice and vegetables.

As shown in Table 5, the per cent infected and the egg counts are higher in the older age groups. This gradual increase in incidence and intensity of infection during the years of childhood is in agreement with observations made by previous investigators in different parts of the world.

b) *Rural areas.* As indicated in Table 6, the examination of this group gave 181 (83 per cent) positive for hookworm. Of the 38 negative cases, 16 were under 5 years of age, giving an incidence of 88 per cent positive in those 5 years and over. The average egg count for the infected individuals was of 1,440 eggs per ml. of feces, with the average for the males of 1,630 and that of the females of 1,260. In all age groups the males had a higher number of eggs than the females. Although in Thailand females share the labor in the field with the males, they spend more time than the males in household duties and in rearing of children. This may account for their somewhat lower average counts.

As in the urban group, there is an increase in the incidence and intensity of infection during the years of childhood. The rate of acquisition of infection in

the rural population is rather rapid. By 10 to 14 years of age, children had acquired about two-thirds of the infections of the adults. This is not surprising in view of the fact that in rural Thailand children over 10 years of age are engaged in agricultural pursuits most of the time when they are not in school.

TABLE 4

The relationship between hookworm egg counts and hemoglobin level

EGG COUNT GROUPS	NUMBER EXAMINED	PER CENT	MEAN HEMOGLOBIN
100 to 999	53	55.2	68
1,000 to 4,999	29	30.2	68
5,000 to 9,999	7	7.3	67
10,000, and over	7	7.3	63
Total	96	100.0	

TABLE 5

The relationship between hookworm egg counts and age in the urban area of Cholburi province

AGE GROUP	NUMBER EXAMINED	NUMBER POSITIVE	PER CENT POSITIVE	AVERAGE EGGS PER ML. OF FECES
5–6	42	7	17	970
7–8	130	22	17	1,300
9–10	114	35	31	2,260
11–14	101	32	31	2,330
Total or average	387	96	25	1,980

TABLE 6

The relationship between hookworm egg counts, age groups and sex in the rural areas of Cholburi province

AGE GROUP	NUMBER EXAMINED			POSITIVE						AVERAGE EGGS PER ML. OF FECES		
				Number			Per cent					
	M	F	Tot	M	F	Tot.	M	F	Tot.	M	F	Mean
1–4	18	15	33	11	6	17	61	40	51	310*	150*	250*
5–9	18	14	32	17	13	30	94	93	94	950	610	800
10–14	20	13	33	19	12	31	95	92	94	1320	1200	1270
15–29	20	19	39	17	15	32	85	79	82	1950	1760	1860
30 and over	29	53	82	25	46	71	86	87	87	2680	1460	1890
Total or average	105	114	219	89	92	181	85	82	83	1630	1260	1440

* These figures show a 50% reduction of counts for stool size in children 1 to 4 years old.

VI. PARASITIC INFECTIONS AND HEALTH STATUS

a) *Height and weight.* In view of the frequent statement in the parasitological literature that physical growth is usually retarded by helminthic infections,

all children examined in the city of Cholburi were accurately measured and weighed.

A perusal of Table 7 shows that in all age groups the children with helminth eggs in their stools at the time of examination were of somewhat lower height and weight than those found to be negative. The differences in each age group are often not statistically significant in view of the great individual variability in height and weight of the children examined. However, considering that in all of the age groups both males and females were of lower height and weight than their unin-

TABLE 7

The relationship between helminthic infection, height and weight in children

AGE GROUP	EGGS IN STOOLS	NUMBER EXAMINED			HEIGHT IN CENTIMETERS		WEIGHT IN KILOGRAMS	
		Male	Females	Total	Males	Females	Males	Females
5–6	Found	16	17	33	107.7	107.7	17.3	16.7
	Not found	6	3	9	108.3	113.3	17.7	19.0
7–8	Found	43	43	86	113.3	113.5	19.1	19.1
	Not found	25	19	44	114.8	114.0	19.9	19.3
9–10	Found	41	27	68	123.8	121.5	23.3	22.5
	Not found	23	23	46	125.0	121.8	23.7	22.7
11–14	Found	40	28	68	133.0	131.5	27.1	26.1
	Not found	18	15	33	134.5	134.5	29.2	29.2

TABLE 8

The relationship between intestinal helminthic infections and the differential leucocytic count

RESULTS OF STOOL EXAMINATIONS	NUMBER EXAMINED	PER CENT OF LEUCOCYTES				
		Neut.	Lymph.	Bas.	Mon.	Eos.
Children without helminths	132	56.7	35.0	0.5	1.8	6.0
Children with hookworm	37	58.9	32.4	0.4	1.4	6.9
Children with *A. lumbricoides*	123	55.9	34.7	0.6	1.6	7.2
Children with *T. trichiura*	12	56.4	32.8	0.3	2.0	8.5
Children with mixed infections	83	56.7	31.3	0.4	1.4	10.2
Total children	387	56.8	33.6	0.4	1.7	7.4
Total adults	60	62.4	32.0	0.5	1.5	3.6

fected controls, the probability that it would happen by chance alone is $1:2^8$, equal to 1 in 256 times, or 0.0038. This figure is regarded as statistically significant.

On the basis of these results one cannot conclude whether physical growth was retarded by the helminthic infections or whether the children of poor families with lower hygienic standards and less adequate nutritional status had greater exposure to infection and/or a lower ability to develop an effective immunity to it.

b) *Differential blood counts.* Blood counts were done on all of the 447 urban patients included in this study. As indicated in Table 8, no consistent differences

were found in the various groups with respect to the percentage of neutrophiles, lymphocytes, basophiles, and monocytes. On the other hand, the percentage of eosinophiles seemed to be influenced by helminthic infections. The proportion of eosinophiles is normally 3 or 4 per cent of the total white cell count. Children without helminths had a mean eosinophilic count of 6 per cent. Those infected with hookworm had 6.9; those with *Ascaris*, 7.2; those with *Trichuris*, 8.5; and those with mixed infections, 10.2. The adult Provincial officers whose degree of helminthic infection was comparatively small had a percentage of eosinophiles of 3.6.

The physical examination of all the people in this series revealed a high incidence of infection other than helminthic among the children. In particular, scabies, trachoma, ear and throat infections were the most prevalent. These diseases were practically absent among the adult Provincial Officers. Although

TABLE 9

The relationship between intestinal helminthic infection, oral temperature, pulse rate, respiration rate and blood pressure

HELMINTH INFECTION	NUMBER EXAMINED	MEAN ORAL TEMPERATURE IN C°	MEAN PULSE RATE	MEAN RESPIRATION RATE	MEAN BLOOD PRESSURE IN MM. OF MERCURY	
					Systolic	Diastolic
Children positive	225	37.4	90	21	92	53
Children negative	132	37.5	74	22	88	55
Total children	387	37.4	84	21	91	54
Total adults	60	36.7	78	20	117	75

the complete findings of the medical examination will be reported elsewhere, it may be stated here that it is believed that some of these infectious diseases are responsible for the rather high percentage of eosinophiles among the children without intestinal helminths.

c) *Temperature, pulse, respiration, and blood pressure.* A perusal of Table 9 indicates that infection with helminths did not alter in any demonstrable way the mean oral temperature, respiration rate and blood pressure of the people examined. The average pulse rate was higher in the infected group, but individual variations were so great as to render this finding of little or no significance at all. It may be noticed, however, that the temperature in children was somewhat elevated. Not infrequently, children with an oral temperature above 38°C. were encountered. It seems not unlikely that the high proportion of throat and ear infections among the children examined may be partly, at least, responsible for their pyrexia.

The blood pressure in all groups was somewhat lower than the average compiled from a quarter million healthy Americans. Normal children around 10 years of age in the United States have a mean systolic pressure of 103 and a mean diastolic pressure of 70 (Best and Taylor, 1945). The children examined in Cholburi had a blood pressure of 91 and 54, respectively.

SUMMARY

A study was made in Cholburi Province, Thailand, to correlate parasitic infections with the general health status. Stool, blood, urine, and physical examinations were done on 387 school children and 60 adult government officials in urban areas. In addition, stools of 219 people from rural areas were examined for helminths.

In the urban population, *Ascaris* was the prevalent parasite in both adults and children. In the rural population, hookworm infection predominated at all ages. In all the places studied, there was a gradual increase in incidence of hookworm and a decrease in incidence of *Ascaris* infection up to 14 years of age. An increase with age in number of hookworm was also found by egg-counts made on a representative sample.

Children with positive stools had a mean lower height and weight than those found to be negative. The differential leucocyte count showed no consistent differences except for a marked eosinophilia in all types of helminth infection. Scabies, trachoma, ear and throat infections were common among children but virtually absent among adult Provincial officers. No obvious relationship was observed between parasitic infections, oral temperature, pulse rate, respiration rate and blood pressure.

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ACQUIRED RESISTANCE TO *SCHISTOSOMA MANSONI* INFECTION IN LABORATORY ANIMALS

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There has long been evidence from serological studies that man and other animals produce antibodies to schistosomes as a result of infection with these parasites. Nevertheless, the body of experimental evidence dealing with actively acquired resistance to the schistosomes is very small. Fujinami's (1916) report was apparently the first. He found that an infected horse was resistant to reinfection with *Schistosoma japonicum* whereas two uninfected horses were highly susceptible. Fujinami and Sueyasu (1917), Tanaka (1926), Ozawa (1930), and Vogel (1949) also found evidence of acquired resistance to *Schistosoma japonicum* in various laboratory animals. Fairley *et al.* (1930) demonstrated that *Schistosoma spindale* will enter monkeys but dies there before maturing, and that this experience confers resistance on the monkeys. Kagan (1951) has presented in abstract form the results of experiments in mice with *Schistosomatium douthitti*, a parasite of small rodents, in which he found that the mice acquired resistance to reinfection.

Two recent reports have dealt with actively acquired resistance to infection with *Schistosoma mansoni*. Watts (1949) concluded that injection into mice of an antigen prepared from *S. mansoni* adults gave the mice a degree of protection against infection. Stirewalt (1951) observed that when larvae of the initial infection were still in the skin of mice, penetration of additional worms was inhibited. She also remarked that when the mouse harbored a matured infection with *S. mansoni* there was an apparent reduction in the number of worms developing from a superimposed infection.

In view of the relatively meager information now available on actively acquired resistance to schistosomes, it seemed desirable to investigate the problem further. Accordingly, an attempt was made to learn whether a measure of acquired resistance to *Schistosoma mansoni* could be produced in laboratory animals. Results of this study are presented here.

MATERIALS AND METHODS

Schistosoma mansoni derived from Puerto Rican stock and maintained in laboratory-reared *Australorbis glabratus* and Swiss mice were used in all experiments but one. The mice used in the experiments were from four strains: Noninbred Swiss, C57BL/6JN (C57 bl subline 6), BALB/cAN (strain c), and A/LN

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(strain A, subline Lilly). All the animals of a given experiment were of essentially the same age and size and were usually females. In two experiments hamsters of both sexes were used.

Each mouse was exposed by immersing its tail in a cercarial suspension according to the method of Olivier and Stirewalt (1952). The hamsters were exposed by placing the cercariae in glass cylinders taped to the clipped belly skin. Only freshly shed cercariae were used and, in all but three instances, the cercariae used in each experiment were the pools shed from 20 or more snails and were of both sexes. In experiments 5, 5a, and 5b the cercariae came from 10 snails and were all males. These single-sex (single miracidium) snail infections were provided through the kindness of Mr. W. B. DeWitt.

The perfusion method of Yolles *et al.* (1947) was used for recovery of worms from the portal system. This method is dependable, consistent and fast. Moreover, it is the only way known to the writers for recovering very small worms from the portal system. In the experiments to be described, worms no larger than the body of *S. mansoni* cercariae were recovered repeatedly from the portal system.

In order to collect worms from the lungs all lobes were removed intact, teased into small bits with sharpened dissecting needles and set aside in 5 ml. of physiological saline for two hours at 37.5°C. The suspension was then diluted with an equal volume of distilled water and the sediment examined thoroughly for worms (Olivier, 1952).

EXPERIMENTS

One way to study acquired resistance to worm infection is to determine whether the existence of a low-grade infection in an animal will protect that animal against the adverse effects (e.g., prevent or delay death) of a superimposed infection. This method can demonstrate the existence of resistance but gives no evidence as to the mechanisms involved. Another way to study the problem is to determine whether, as a result of previous infection, fewer worms of a new infection are able to survive.

The first of the two methods was used in the experiments presented in Table 1 and the second method was used in the experiments of Table 2. In three instances both methods of study were applied simultaneously to a single animal population. Thus, experiment 3 (Table 1) and experiment 3a (Table 2) might be considered two parts of the same experiment since they were done simultaneously using the same animal population and differ only in that some of the challenged animals were autopsied after 28 to 30 days so that worm counts could be made, while the remainder were kept until they died. These two experiments can be compared closely, and the differential worm counts from experiment 3a give an estimate of the 28 to 30 day level of worm populations in the mice of experiment 3 which later died of the infection.

In the same way experiments 4 and 4a are part of the same experimental series and experiments 5, 5a, and 5b are also part of one experimental series.

TABLE 1

The effect of preexisting infection with Schistosoma mansoni *on the time of death of mice given a large challenge exposure to the same parasite*

EXP.		NUMBER OF MICE SURVIVING FROM INITIAL GROUP		NUMBER OF CERCARIAE GIVEN PRIOR TO CHALLENGE	DURATION OF INFECTION BEFORE CHALLENGE (WKS.)	NUMBER OF CERCARIAE IN CHALLENGE EXPOSURE	DEATHS BY WEEKS AFTER CHALLENGE																AVERAGE LENGTH OF LIFE AFTER CHALLENGE (WKS.)	STANDARD DEVIATION
							Weeks																	
							1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
1	Superinfected	41	Swiss	25 + 25	16	529	0	0	3	3	0	1	0	5	12	8	2	3	0	1	1	0*	9.2	3.35
	Controls	35		0	0		1	0	0	0	0	1	1	17	12	3	—	—	—	—	—	—	8.2	1.48
2	Superinfected	65	Swiss	25	13	583	1	1	0	0	0	2	2	5	11	9	9	9	5	2	2	2†	10.9	3.08
	Controls	70		0	0		0	0	0	0	1	0	2	39	13	8	3	3	1	—	—	—	8.7	1.31
3	Superinfected	39	Swiss	25 + 30	16	360	0	2	2	0	2	1	0	7	3	12	3	4	2	1	—	—	9.0	2.97
	Controls	42		0	0		0	0	0	0	0	0	2	17	11	9	1	1	1	0	—	—	8.9	1.22
4	Superinfected	46	A/LN	25 + 30	13		3	0	0	4	2	7	14	5	3	3	1	4	0	—	—	—	7.1	2.66
	Controls	42		0	0		0	0	0	0	0	3	15	15	8	1	0	0	0	—	—	—	7.7	.93
5	Superinfected	38	C57BL/6JN	45‡	16	348	0	0	0	0	0	0	0	23	14	0	1	—	—	—	—	—	8.5	.64
	Controls	36		0	0		0	0	0	0	0	0	0	25	8	3	—	—	—	—	—	—	8.4	.67

* Two survivors after 16 weeks.

† Five surviving after 16 weeks.

‡ Male cercariae only.

TABLE 2

Tests for resistance to Schistosoma mansoni *in mice and hamsters as measured by worm counts*

PART 1. *Experiments based on worm counts from the portal system*

EXP.	HOST			NUMBER OF CERCARIAE GIVEN PRIOR TO CHALLENGE	DURATION OF INFECTION BEFORE CHALLENGE (WKS.)	NUMBER OF CERCARIAE IN CHALLENGE EXPOSURE PER HOST	NUMBER OF DAYS BETWEEN CHALLENGE AND AUTOPSY	NUMBER OF ANIMALS PER GROUP		AVERAGE NUMBER OF OLD WORMS AT AUTOPSY	AVERAGE NUMBERS OF NEW WORMS AT AUTOPSY (± STANDARD ERROR OF MEAN)
	Aminal	Strain	Sex								
3a	Mouse	Swiss	♀	25 + 30	16	360	28–30	26	Superinfected	3.8	54.0 ± 4.8
						360	28–30	20	Controls	—	88.2 ± 4.0
4a	Mouse	A/LN	♀	25 + 30	13	339	22–28	20	Superinfected	3.1	20.9 ± 7.3
						339	22–28	20	Controls	—	97.8 ± 6.1
5a	Mouse	C578L/6JN	♀	45*	16	348	30	10	Superinfected	18.5	73.5 ± 8.7
						348	30	10	Controls	—	101.8 ± 10.4
5b	Mouse	C578L/6JN	♀	45*	16	348	35	10	Superinfected	16.3	75.6 ± 6.5
						348	35	8	Controls	—	101.5 ± 9.0
6	Mouse	Swiss	♀	20 + 25	41	318	27–28	16	Superinfected	9.9	26.6 ± 3.4
						318	27–28	18	Controls	—	32.8 ± 2.6
7	Mouse	C578L/6JN	Mixed	100	21	239	24	5	Superinfected	48.6	6.6 ± 4.1
						239	24	6	Controls	—	85.3 ± 7.4
8	Mouse	Swiss	♀	112	18	239	27	4	Superinfected	20.0	17.5 ± 5.7
						239	27	8	Controls	—	60.9 ± 3.9
9	Mouse	A/LN	♀	50 + 25	12	238	22–23	6	Superinfected	14.3	0.5 ± 1.0
						238	22–23	10	Controls	—	41.1 ± 3.0
10	Mouse	Swiss	♀	15 + 20 + 20	29	352	17	4	Superinfected	13.0	14.2 ± 6.1
						352	17	10	Controls	—	29.7 ± 4.1
11	Hamster		Mixed	100 + 50	6	100	18	6	Superinfected	77.0	12.7 ± 2.5
						100	18	9	Controls	—	27.8 ± 5.2
12	Hamster		Mixed	100	5	100	21–22	13	Superinfected	57.6	11.5 ± 2.9
						100	21–22	9	Controls	—	31.1 ± 5.1

PART 2. *Experiments based on worm counts from the lungs*

9a	Mouse	A/LN	♀	50 + 25	12	238	7	10	Superinfected	17.2	10.0 ± 2.0
						238	7	10	Controls	—	41.0 ± 3.5
10a	Mouse	Swiss	♀	15 + 20 + 20	29	352	6	5	Superinfected	15.0	6.8 ± 2.6
						352	6	5	Controls	—	32.0 ± 4.8

* Male cercariae only.

The effect of preexisting infection with Schistosoma mansoni *on the ability of mice to tolerate heavy reexposure*

Five experiments were conducted in order to test whether a light infection with *S. mansoni* in mice could protect them against the effects of an exposure known to be lethal to normal mice.

A preliminary experiment was first conducted in order to learn the number of cercariae required to kill a normal mouse. Forty-two Swiss mice were divided into five groups, with 10 in one group and 8 in each of the four others. All groups were then exposed to various concentrations of cercariae from a single pool so that mice received exposure to the following numbers of cercariae: group 1 (10 mice) 137 cercariae each; group 2, 274 cercariae; group 3, 548 cercariae; group 4, 720 cercariae; and group 5, 1440 cercariae.

Except for one animal (5 weeks), there were no deaths before the seventh week after exposure. The average time of death for 9 of the mice of group 1 was 19 weeks after exposure, and the remaining mouse was still alive after 29 weeks. In group 2 the average life after exposure was 10 weeks and all were dead after 16 weeks. In the other three groups the average survival time after infection was 8 weeks, and all the mice were dead in 10 weeks. This experiment suggests that 500 cercariae, or perhaps even less than this, will kill normal mice and that even tripling the number of cercariae (from 500 per mouse to 1,440) does not materially change the average time of death nor does it advance the time of the first death. On the basis of this experiment cercarial exposures ranging in size from about 339 to 600 per mouse were used to challenge the mice in the experiments to be described.

Five experiments tested the effect of a light infection on the tolerance of mice for a heavy exposure to *S. mansoni*. The mice were divided into two groups, and one was given a light infection while the other was left uninfected. In experiments 1, 3, and 4 the initial light exposure was repeated after 2 to 6 weeks. After an interval of 13 to 16 weeks the survivors of all groups previously exposed to cercariae as well as the uninfected group were then challenged with a heavy exposure to cercariae and the date of death of each mouse recorded. In the first four experiments the early exposures were to cercariae of both sexes, but in the fifth only male cercariae were used and therefore no females or eggs were present at the time of the challenge exposure. Data from these experiments are presented in Table 1.

In the first four experiments most of the controls died during a relatively short space of time in the seventh, eighth and ninth weeks after the challenge. On the other hand, deaths in the superinfected groups were spread out with only a low peak during the seventh to twelfth weeks. Some of the other deaths came before the peak of deaths among the controls, and some came long after all the controls were dead. Therefore, although the differences in average survival time between superinfected and control mice in these experiments are not statistically significant, nevertheless, the two groups differed significantly in their survival patterns. A statistical test based on Bartlett's test (Snedecor, 1946, p. 249) for homogeneity gave highly significant chi squares in the first four experiments. The varia-

tion in time of death in preinfected animals was significantly greater than for the control animals.

In the fifth experiment there was obviously no difference between the two groups either in average survival time or in the over-all pattern of survival.

The effect of preexisting infection with S. mansoni *on the number of worms resulting from a superimposed infection*

Thirteen experiments (Table 2) were designed to test whether the existence of infection with *S. mansoni* in mice and hamsters protected the animals against reinfection. In every case a homogeneous group of young animals was divided into two lots. One of the lots was then exposed to a small number of cercariae. The number used was sufficiently large to infect all animals but small enough so that only a small proportion of the infected animals died as a result of this infection. In six of the experiments the initial light exposure was repeated after 2 to 6 weeks. In one experiment three light exposures were given.

The infected animals and their controls were then set aside and after a suitable period, usually many weeks, all were exposed to cercariae of *S. mansoni* simultaneously. In the mouse experiments the animals were allowed to go from 12 to 41 weeks before they were challenged. In the hamster experiments the infections in the test groups were 5 and 6 weeks old at the time of challenge. Each animal was challenged by exposing it to from 239 to 360 cercariae. This number was large enough to provide substantial numbers of worms in the control animals.

From 17 to 35 days after the date of the challenge exposure, the animals in 11 of the experiments were examined for worms by perfusing the portal system (Table 2, Part 1). Previous experience had shown (Olivier, 1952) that the worms can be recovered from the portal system as soon as they arrive there; that the first worms reach the portal system in about 10 days; and that most of the worms destined to reach the portal system do so by the end of the third week. The interval between challenge and autopsy could be no longer than 30 or 35 days, since after that time worms of the challenge infection are so large that they cannot be distinguished with certainty from worms already present at the time of challenge.

All worms found in the portal system were counted, and those from the superinfected animals were classified as to whether they were "old," that is, present before challenge, or "new," that is, derived from the challenge exposure. The "old" worms could usually be distinguished readily from the "new" ones since they were large, obviously mature, usually in copula and located principally in the mesenteric portion of the portal system which was perfused separately from the hepatic portion. The "new" worms were smaller, immature, unpaired and were predominantly in the liver. All worms that could not be put in the "old" group with certainty were classified as "new."

Worm counts were made from the lungs of the animals in the experiments reported in Part 2 of Table 2. These two experiments (9a and 10a) were done in conjunction with experiments 9 and 10 respectively and are comparable with them except for the time and method of examination. The worms were sought

in the lungs 6 and 7 days following exposure, since Olivier (1952) had shown that although no worms reach the portal system by the end of the first week, they can be recovered from the lungs at that time.

From the data presented in Table 2, it is apparent that in all 13 experiments the average number of new worms in the superinfected animals was less than that in the controls. This pattern is highly significant statistically. Using a crude statistical test, the "signs" test, the probability that the results of all of 13 experiments will be in the same direction is less than $(\frac{1}{2})^{12}$. However, a detailed statistical analysis was carried out using the square roots of the numbers of worms observed. This modification of the data was made because the usual statistical tests (Student's t, Analysis of variance, etc.) require that the data be normally distributed and that the groups compared have equal variances. Data such as the counts reported here are probably distributed in accordance with the Poisson law and the square root transformation casts such data into the theoretically acceptable form (Snedecor, 1946, p. 446). The analysis of the differences of the average counts of organisms in the 13 experiments shown in Table 2 led to a value of Student's t of 4.86 with 12 degrees of freedom. For such a t, P is $<.001$.

Further statistical analysis showed that there is no relation between the number of old worms found in the superinfected animals and the number of new worms found in these animals. In the 13 experiments there were 7 positive correlations (larger numbers of old worms associated with larger numbers of new worms) and 6 negative correlations (larger numbers of old worms associated with fewer new worms). No significant relation, overall, between the numbers of old worms and the numbers of new worms could be shown. For the common, overall regression we found an F ratio of slightly less than 1 with 1 and 109 degrees of freedom.

Furthermore, in the mouse experiments, there is no relation between the average number of old worms in any experiment and the magnitude of the average difference between control and superinfected animals. (Student's $t = 1.03$ with 9 degrees of freedom.) This, in part, is a further examination of the supposition that there might be some relation between the number of old worms and the number of new worms. This comparison was not made for the hamster infections since there were only two experiments.

DISCUSSION

In all the experiments dealt with in Part 1 of Table 2, the superinfected animals harbored, on the average, fewer worms of the challenge exposure than did the controls. These differences are statistically significant and one must conclude that the presence of worms in the portal system of the host was accompanied by a reduction in the number of new worms that reached the portal system.

Since there was no relation between the number of old worms present in the superinfected animals and the number of new worms found, one cannot assume that this reduction in the number of new worms is due to lack of space or to crowding. It seems reasonable to conclude that the presence of the old infection

caused the activation in that host of a defensive mechanism which effectively reduced the number of new worms that could reach the portal system.

The two experiments in which the worms were collected from the lungs show that the number of new worms in superinfected animals is significantly reduced as early as the first week after reinfection when they are still migrating. Apparently the defensive mechanism is effective before the worms reach the lungs or takes effect immediately after they get there. When experiments 9 and 10 are compared with 9a and 10a, it is interesting to note that although the numbers of worms recovered from the controls were about the same 7 days after challenge as they were 22 to 23 days after challenge, the counts of new worms from the superinfected animals are not as consistent. Explanation of this apparent difference is impossible on the basis of the available data, but it may possibly be attributed to differences in the intensity of the immune response from mouse to mouse in the superinfected groups.

Results of the first four survival experiments are interpreted as showing that the presence of a previous bisexual infection has a significant influence on the survival pattern of the mice. In the control mice the infections ran a typical course with almost no deaths until there was a crisis in the seventh and eighth weeks resulting in the death of almost all the animals. In the superinfected mice there were scattered deaths during the first six or seven weeks after challenge. Perhaps these should be attributed to damage caused by the developing new infection in mice already weakened by their parasite burden and unable to tolerate the additional worm burden. Deaths in the superinfected animals were most numerous in the seventh to the tenth or twelfth weeks, but some of the mice survived for many weeks longer. Apparently, the latter mice were sufficiently immunized by the previous infection so that they were able to live longer, following the challenge, than the controls. Thus, the existence of previous infection seems to modify the survival pattern by greatly spreading out the time of death of the mice.

In the experiments in which the first infection consisted of only male worms, there was evidence that the single sex infection conferred a measure of acquired resistance in the mice since the average worm counts were lower in the superinfected animals than in the controls. Yet the survival pattern of the mice with these previous male schistosome infections was not altered significantly from that of the controls. Apparently the worm reduction encountered in those superinfected mice was not sufficient to affect the chances of survival of the mice in the presence of the new infection.

On the other hand, the pattern of survival of these superinfected mice with single sex infections does appear different from that of the superinfected mice with mixed sex infections, in that there were no early or delayed deaths in the former group. This may be interpreted as indicative of a lack of any immune response due to lack of egg production stimulating the immune mechanism. The immune reaction itself in animals with mixed sex infections may have been responsible, on an allergic basis, for causing early deaths and, on a resistance basis, for the prolongation of survival.

SUMMARY

An attempt was made to demonstrate acquired resistance to *Schistosoma mansoni* infection in mice and hamsters. In one series of experiments, mice with light bisexual *S. mansoni* infections were challenged with an exposure great enough to kill uninfected mice. The survival pattern of the superinfected mice was significantly different from that of the controls in that the deaths were spread out over a much longer period. Apparently some of the mice were sufficiently immunized so that they were able to survive longer than the controls. In another set of experiments infected mice and hamsters were similarly challenged. Later, total worm counts were made and it was found that significantly fewer new worms developed in the superinfected animals than in the controls. These findings demonstrate that mice and hamsters develop resistance to reinfection with *S. mansoni*.

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THE EFFECT OF A CYANINE DYE ON INFECTIONS WITH HOOKWORM AND TRICHURIS IN MAN[1]

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Studies described in a previous paper (Hales and Welch, 1952) indicate that certain cyanine dyes have anthelmintic properties and that these compounds may be useful in the treatment of certain parasitic infections. A group of these dyes, particularly 1′-ethyl-3,6-dimethyl-2-phenyl-4-pyrimido-2′-cyanine chloride, is effective in the treatment of filariasis of the cotton rat caused by *Litomosoides carinii* (Welch *et al.*, 1947; Peters *et al.*, 1949). A closely related compound, ✱715 (Hales and Welch, 1952), exerted marked chemotherapeutic activity in canine ascariasis, trichuriasis and ancylostomiasis, but was without value in the treatment of canine tapeworm infections. This study (Hales and Welch, 1952) showed that the compound produces some anorexia and occasional vomiting when administered to dogs, and a mild renal tubular change of a reversible nature as the only manifestation of pathologic change due to the oral administration of the drug in the dosages found effective chemotherapeutically. Toxic reactions observed during administration of *large* doses of this drug to human subjects are epigastric pain, nausea and vomiting and a marked laxative effect. The last reaction may be an advantage in its use as an anthelmintic agent.

Since the drug ✱715 proved effective in clearing hookworms from dogs, its effect on human hookworm infection was investigated. Six patients suffering from severe infection with hookworms were hospitalized at the University Hospital of the School of Tropical Medicine for treatment with this drug. All the patients were indigent males with moderate to severe hypochromic microcytic anemia. The youngest patient was 9 and the oldest 61 years old; five of the patients were under 20 years of age. Their weight varied from a minimum of 22.5 kg. (patient A-13048) to a maximum of 53 kg. (patient A-12953).

The minimum dosage of the drug given was 9 mgm. per kgm. of body weight per day, while the maximum daily dose was 27 mgm. per kgm. The minimum total dosage given was 0.35 gm. (patient A-13048), and the maximum total dose 3.6 gm. (patient A-12953). With the exception of patient A-13048, who was treated only once, all the patients were treated with ✱715 at least twice.

The drug was usually given in divided portions throughout a 24-hour period, although single doses were used on three occasions. One patient (✱13285) received equal amounts of the drug every 8 hours for 48 hours (see Table 1). A total of fifteen courses of treatment were given to these six patients, using different schedules of administration and various doses of the drug.

[1] We are grateful to Parke, Davis and Company for an adequate supply of the cyanine dye (✱715) in encapsulated form.

Subsequent to treatment with # 715 and the determination of its effect, all the patients except # A-12953 were given tetrachlorethylene in appropriate amounts for age and weight. This was done in order to obtain any worms which were not expelled during treatment with # 715.

Following drug administration all bowel movements were collected and washed and all worms were recovered and counted. The percentage recovery of worms after treatment of the patients with # 715 and with tetrachlorethylene was thus determined. Determinations of the number of eggs per gram of feces were made previous to and during the administration of the drugs.

RESULTS

Reactions to drug. Many undesirable though no serious signs or symptoms were encountered during treatment with # 715. The most distressing disturbances were epigastric pain, nausea and vomiting. Nausea occurred in 10 of 16 instances in which the drug was used; vomiting and epigastric pain were present on six occasions and occurred from one-half to six hours after medication. The vomitus was usually described as pink-colored, a circumstance attributable to the red color of the compound since the presence of blood was excluded by appropriate tests. Epigastric pain was usually of moderate intensity, but it was quite severe in case A-13024, especially during the second and third treatments. Diarrhea of mild nature occurred in all cases. Anorexia was complained of on six occasions, but appetite returned in all patients soon after treatment was terminated. A rather indefinite sensation of dizziness was complained of on five occasions, but it also disappeared soon after treatment was finished. A temperature of 38.3° C. developed in patient A-13285 and lasted for approximately 8 hours when the drug was given on two consecutive days. Whether the fever was attributable to the drug could not be determined. No definite relation could be determined between dosages of the drug given or mode of administration and the development of untoward symptoms.

During treatment there were no alterations from the normal pattern in the laboratory tests performed, which included complete blood counts, differential leucocyte counts, urinalysis, nonprotein nitrogen, urea nitrogen, creatinine and the Hanger test.

Anthelmintic effect. The stools collected after administration of # 715 were either diarrheic or mushy, and cherry colored. The worms recovered were dead and tinged slightly red, but were in a well-preserved condition.

The table shows the number of hookworms and trichuris passed after the administration of drug # 715 and of tetrachlorethylene. Although the number of worms passed with drug # 715 was considerably less than that recovered after the administration of tetrachlorethylene, it is evident that the former drug possesses a definite antiparasitic effect. Furthermore, as the table indicates, more worms were expelled upon continued administration of the drug. It is of interest that adult *Trichuris trichiura* also were passed after treatment with # 715. The effect of this drug on this infection is being investigated further.

The cyanine dyes are a new class of compound with antiparasitic properties

TABLE 1

Dosage, toxicity and properties of # 715

PATIENT NO.	AGE	WT. IN KGM.	DOSE MGM./ KGM. PER 24 HR.	TOTAL MGM./ KGM.	MODE OF ADMINISTRATION	REACTIONS TO DRUG						WORMS RECOVERED AFTER EACH DRUG								
												# 715					Tetrachlorethylene			
												Hookworm				Trichuris	Hookworm			Trichuris
						N	V	Ep.	D	Di.	A	Males	Fem.	Total	% Recov.	Total	Males	Fem.	Total	Total
A-13048	9	22.5	15	350	117 mg. t. i. d. p.c.	+	0	+	0	+	0	60	17	77	10.6	0	224	420	644	4
A-13244	16	33	18	600	200 mg. t. i. d. fasting	0	0	0	0	+	0	128	39	167	12.1	9	466	744	1210	0
			22.5	1350	250 mg. t. i. d. p.c.	+	0	+	0	+	+									
A-13285	19	50	20	2000	333 mg. t. i. d. q. 8h. (48 hr.)	0	0	0	+	+	0	88	39	127	31.0	1	121	261	382	0
			20	3000	333 mg. t. i. d. q. 8 h. (72 hr.)	0	0	0	+	+	0									
A-13024	17	27	11	300	300 mg. fasting	+	0	0	+	+	+	294	545	839	37.9	0	539	884	1423	0
			22.5	600	200 mg. t. i. d. p.c.	+	+	+	+	+	+									
			22.5	1500	200 mg. t. i. d.	+	+	+	+	+	+									
A-12526	14	28	18	500	250 mg. t. i. d.	+	+	0	0	+	+	273	263	536	79.7	15	4	132	136	0
			27	750	250 mg. t. i. d. p.c.	+	+	+	0	+	+									
			9	250	250 mg. fasting	0	0	0	0	+	0									
			11	1800	300 mg. fasting; 300 mg. t. i. d. p.c.	+	+	0	0	+	0									
A-12953	61	53	22.5	1200	400 mg. t. i. d. p.c.	+	0	+	0	+	0	71	37	108			Not administered			
			22.5	1200	400 mg. t. i. d. p.c.	0	0	0	0	+	0									
			22.5	3600	400 mg. t. i. d. p.c.	+	+	0	0	+	+									

N—nausea; V—vomiting; Ep. epigastric pain; D—dizziness; Di—diarrhea; A—anorexia.

which deserve further study. Unlike some of the anthelmintic drugs in use today, the toxicity of the cyanine dyes is only temporary and their use in human subjects has revealed no evidence of tissue damage. It is possible that through further investigative work more effective, and better tolerated compounds of the cyanine group may be found.

SUMMARY

The administration of compound #715 to patients infected with hookworm caused expulsion of some of the adult worms. As the doses and frequency of administration increased, the number of worms expelled also increased, but it was never as effective nor as well tolerated as tetrachlorethylene. Adult *Trichuris trichiura* also were passed during administration of #715.

Frequent complaints during drug administration were nausea, vomiting, and epigastric pain. These reactions, however, did not warrant discontinuation of treatment.

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CURRENT STATUS OF INSECTICIDE RESISTANCE IN INSECTS OF PUBLIC HEALTH IMPORTANCE

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INTRODUCTION

A year ago the author gave a summary of the significance of insecticide resistance in vector control programs in various parts of the world (Hess, 1952). The present paper reviews some of the new developments on insecticide resistance in insects of public health importance which have come to the author's attention during the past year. The terminology used is the same as that adopted in last year's paper.

On December 8–9, 1951, a conference on insecticide resistance and insect physiology was held in Cincinnati. At the request of the Army Medical Research and Development Board, the conference was sponsored by the Division of Medical Sciences of the National Research Council. The published results (National Research Council, 1951) summarize the current status of information on insecticide resistance and outline some of the problems for the future. Following the conference, recommendations were made for realignment and extension of research on insect physiology and insecticide resistance including investigations on the mechanism and genetics of resistance, the mode of action of insecticides and fundamental studies on insect biology, ecology, and physiology. Subsequent to the meetings, the U. S. Army Surgeon General initiated action to establish a research program providing for investigation of the various problems relating to insecticide resistance to extend over the next several years. A number of projects have already been approved and investigations are under way which should provide much useful information for the solution of the resistance problem. This stimulus for fundamental research is in keeping with recommendations of the Expert Committee on Insecticides and on Malaria of the World Health Organization (Expert Committee on Malaria, 1951). The U. S. Public Health Service, including the National Institutes of Health and the Communicable Disease Center, is also increasing fundamental research on insecticide resistance. There is increasing recognition that replenishing of our reservoir of fundamental knowledge will in the long run provide a much more effective solution to the resistance problem than did the trial and error use of insecticides in the past. It is hoped that this attitude will be given increasing support during the next few years.

CURRENT STATUS OF RESISTANCE

House flies

High levels of resistance to DDT and other chlorinated hydrocarbons continue to be exhibited by house flies in most parts of the world, and appear to develop wherever there are extensive applications of residual and/or larvicidal sprays of these materials. India and Ceylon continue to be the one exception, *Musca*

vicina and *Musca nebulo* still exhibiting no significant resistance even where extensive residual spraying programs have been carried out for a number of years (Jayewickreme, 1952). Pal *et al.* (1952) have shown in laboratory studies that *M. nebulo* can develop some resistance to DDT but not to the same degree as does *M. domestica*. Although *M. vicina* has not become resistant in India and Ceylon, it does exhibit high levels of resistance in the Near East and Africa. Both physiological and behavioristic types of resistance have been reported for this species in Israel (Silverman *et al.*, 1952).

In the more northern part of the United States and in Canada DDT-resistant house flies continue to be localized. In all cases which have been investigated in Canada, complaints of noneffectiveness originate from areas where DDT has been extensively used during the past few years (Roadhouse, 1952). Lindane has provided a satisfactory substitute in some areas.

Dilan has continued to show some promise as a substitute for chlorinated hydrocarbons in the control of resistant house flies, although laboratory studies have shown that house flies may develop a resistance to Dilan constituents to as high a level as to DDT (March *et al.*, 1952). The use of various types of synergists and of certain organic phosphorus compounds offer the other major lines of promise for effective chemical control. The Technical Development Branch of the Communicable Disease Center has obtained very promising results with parathion impregnated strings festooned below the ceilings of dairy barns (Maier *et al.*, 1952).

Anopheline mosquitoes

The past year has brought additional evidence of the development of insecticide resistance in anopheline mosquitoes. One of the most significant findings has been the discovery by Trapido (1952) that in Panama *Anopheles albimanus* has developed a definite change in its reaction to DDT residual sprays since the time this material was first used. For example, in the town of Gatuncillo, which has received 20 treatments of DDT in the 86 months since the first application in November 1944, the number of *A. albimanus* collected per house per night has changed from 3.0 in 1945 and 1946 to 21.7 in 1952. Although the residual sprays no longer reduce the numbers of mosquitoes in the houses, there has been no significant change in the per cent of engorged mosquitoes resting therein. There has, however, been a change in the 24-hour per cent survival of these engorged mosquitoes, some 35 per cent surviving in 1952 as compared with only 4 per cent in 1945. It is therefore quite apparent that the actual number of human-fed mosquitoes which survive the treatment is much greater than in 1945, with a consequent increase in the malaria transmission potential. Although there has as yet been no return of malaria in the experimental area, this change in the reaction of the mosquitoes certainly suggests dangerous possibilities for a break through the transmission threshold. From his preliminray observations, Dr. Trapido is not sure whether the change is due to an actual acquisition of physiological resistance by *A. albimanus* or to the development of a hyperirritability to DDT, since the adults collected from the treated houses appear to

be much more restless and spend less time sitting upon treated surfaces than previously observed.

It is interesting to note that Gabaldon (1952) has observed that there have been some increases in *A. albimanus* populations in Venezuela but without increase in malaria. There are still differences of opinion with regard to the possible development of behavioristic resistance by *Anopheles darlingi* in South America. Giglioli (1952) feels that it is not possible for resistance to develop in either *A. darlingi* or *Aedes aegypti* because both are so highly susceptible to DDT that eradication in treated areas is rapid and complete; however, he does report (1952A) that malaria is still being contracted by Indians on the forested farms of the interior of British Guiana which, due to their remoteness and shifting character, escape control. Gabaldon (1952) has observed in Venezuela that whereas *A. darlingi* apparently is eradicated in some treated areas, in others it is not. He has suggested that these differences may be related to the degree of domesticity of the strains; that is, those which are more anthropophilic are eradicated whereas the more zoophilic strains may at least partially survive. Pinotti (1951) has observed that whereas *A. darlingi* seems to be disappearing from certain treated areas, in others which have been treated twice yearly with DDT since 1947 the density of this species is still very high. Bustamante (1951) has stated that *A. darlingi* exhibits a higher index of zoophilism in Brazil than it does in British Guiana or Venezuela. He feels that this species may eventually be eradicated in the coastal areas but that eradication probably will not be possible in the central uplands. This type of situation is most favorable for the development of behavioristic resistance, and the author would not be surprised if there are reports of failures of DDT residuals to control *A. darlingi*-transmitted malaria in interior areas of South America in the next year or two.

The observations on *Anopheles sacharovi* in Greece (Hadjinicolaou, 1952; Livadas, 1951) suggest a strong similarity to the situation observed with *A. albimanus* in Panama, much larger number of adult mosquitoes occurring in treated houses than when the program was first initiated. For example, during the past season Livadas (1952) observed considerable numbers of *A. sacharovi* in dwellings in the village of Asterion the day after they were sprayed with DDT; in the first years of spraying the catching of a few anophelines in sprayed quarters even many months after spraying constituted a very rare finding. He reports that *A. sacharovi* collected from sprayed quarters show a high percentage of survival after 24 hours. They also exhibit resistance to Gammexane, but not to dieldrin or chlordan. Some malariologists still feel, however, that the development of resistance by *Anopheles* in Greece is still not significant from a practical standpoint.

There have been reports from field observers that in certain areas of Turkey (Berksan, 1952) *Anopheles superpictus* is developing resistance to DDT.

In Sardinia, *Anopheles labranchiae* still persists at extremely low levels in a few restricted areas (Aitken, 1952). However, it is not clear whether this is due to resistance or to incomplete control. No malaria has been officially reported from the Island, but special surveys during the past season revealed 14 cases, all presumably relapses, 7 of which were *falciparum* (Warren, 1952).

Differences of opinion with regard to the control of *Anopheles minimus flavirostris* in the Philippines have not yet been resolved. Some workers are of the opinion that DDT residual spraying of houses is not an effective malaria control procedure in the Philippines because of the wild nature of the vector (U. S. Public Health Service, 1950). However, other workers have reported successful control of *A. minimus flavirostris* in this area through the use of DDT residual sprays (Morrill, 1952).

In the United States there has been little change in the situation with regard to *Anopheles quadrimaculatus* in the Tennessee Valley area where studies have indicated a definite though slight development of resistance to DDT. Observations by the Bureau of Entomology and Plant Quarantine in Florida also have given definite evidence of the development of low levels of resistance by *A. quadrimaculatus* in areas along the east coast of Florida which have been treated with DDT for a number of years to control salt-marsh mosquitoes (Knipling, 1952).

Culicine mosquitoes

The resistance of salt-marsh mosquitoes (*Aedes sollicitans* and *Aedes taeniorhynchus*) along the eastern coast of the United States has become an increasing problem and more and more complaints of ineffective control are being received from commercial operators and mosquito abatement districts. Hadjinicolaou (1952) has reported the occurrence of DDT-resistant salt-marsh mosquitoes in Greece.

There has been some increase in the problem of resistant culicine mosquitoes in California, particularly in the San Joaquin Valley (Gjullin, 1952). Resistance in *Culex tarsalis* has extended throughout the Kern County district and larvae are now resistant in small districts in Fresno County. *Aedes nigromaculis* has become resistant in one area of the Delta district in Tulare County. EPN (ethyl *p*-nitrophenyl thionobenzenephosphate) has shown promise as a larvicide for the control of these resistant species (Scudder, 1952).

Some observations by Crandell (1952) at Toledo, Ohio, with *Culex pipiens* have indicated that larvae collected from a heavily treated area showed significantly lower mortality when exposed to DDT than did larvae which were collected from untreated areas. This is not surprising in view of experiences with members of the *C. pipiens* complex in various other parts of the world (Hadjinicolaou, 1952; Hess, 1952). However, investigations by Pal *et al.* (1952) gave no evidence of increased resistance in *C. fatigans* in DDT sprayed areas in India.

There have been rumors in the United States of the development of resistance in other species of culicine mosquitoes, such as *Aedes vexans*, but to date the author has been unable to obtain any positive information on such occurrences.

Other species

The demonstration of chlordan resistance in the German roach (*Blattella germanica*) is another important finding of the past year. Field observations by

Bedingfield (1952) at Corpus Christi, Texas, indicated that German roaches in that area were becoming resistant to chlordan. Specimens were collected and distributed by Dr. Heal of the National Pest Control Association to the U. S. Bureau of Entomology and Plant Quarantine where the occurrence of the resistance was confirmed (Babers, 1952). It was found that, whereas only 40 gamma chlordan per gram of roach applied by the measured drop test gave complete mortality of normal female roaches, a dosage of 1280 gamma gave only 40 per cent mortality in the resistant strain. Hadjinicolaou (1952) has reported DDT-resistant cockroaches in Greece, though the species was not named. Grayson (1952) has conducted laboratory experiments demonstrating the ability of the German roach to develop resistance also to DDT and benzene hexachloride.

A report of the development by bedbugs (*Cimex lectularius*) of resistance to DDT in Hawaii was given in last year's report. There has been a subsequent report of the development of DDT resistance by this species in Greece (Livadas, 1951).

The demonstration of high levels of resistance to DDT by body lice in Korea was one of the most important items in last year's review. It has been shown subsequently that body lice in Egypt have developed a degree of resistance in certain areas where periodic dusting with DDT has been carried out every three to five months since 1948 (Hurlbut, 1952). Field tests have indicated that the number of persons infested, or heavily infested, after treatment is definitely greater than was observed in the same area in 1945. It is believed that DDT could still be used effectively in case of a typhus epidemic, but intensive use of DDT might result in a rapid increase of resistance. Evidence has also been reported that lice found on Palestine refugees in the Near East are developing resistance to DDT dusting powders (Wright, 1952).

Resistant human fleas (*Pulex irritans*) were reported last year from Greece and South America, and there is now some evidence that in the Near East also they are developing resistance to DDT (Wright, 1952). In Greece this species has been reported to be "completely resistant" to DDT (Hadjinicolaou, 1952). In the United States, reports are frequently received that cat and dog fleas (*Ctenocephalides*) are developing resistance to insecticides (Mohr, 1952), but no confirmation of this has come to the author's attention. It is probable that if more study were given to the development of resistance by animal ectoparasites, a number of cases of such resistance would come to light. For example, in South Africa the cattle tick, *Boophilus decoloratus*, which was previously resistant to arsenicals, has now developed a high level of resistance to BHC (Whitnall *et al.*, 1952).

SUMMARY

The accompanying table summarizes the current status of resistance to insecticides by insect pests of public health importance in various regions of the world. There has been experimental confirmation of resistance in a number of species, and field observations of resistance are becoming increasingly common. No one who is now concerned with the control of insect pests of public health importance

can fail to see the potential impact of resistance upon control programs. For example, a breakdown of the effectiveness of insecticides in controlling malaria mosquitoes might be quite disastrous in some areas of the world where the loss of acquired immunity during a period of satisfactory control has made the popu-

Current status of insecticide resistant insects of public health importance in various regions of the world

	N. AMERICA	S. & C. AMERICA & CARIBBEAN	EUROPE	AFRICA	NEAR EAST	INDIA & CEYLON	AUSTRAL-ASIA & PACIFIC ISLANDS
House flies							
Musca domestica	Pb	Pb	P				P
M. vicina				Pb	P	O	
M. nebulo						O	
Anopheles mosquitoes							
A. albimanus	P or B						
A. darlingi		b					
A. gambiae				B			
A. maculipennis			p or b				
A. quadrimaculatus	PB						
A. sacharovi (=*elutus*)			p or b				
A. superpictus			p or b				
Culicine mosquitoes							
Aedes caspius			p or b				
A. dorsalis	P						
A. nigromaculis	P						
A. sollicitans	P						
A. taeniorhynchus	P						
Culex pipiens	p		P				
C. quinquefasciatus	p	P					
C. tarsalis	P						
Other species							
Blattella germanica	P		p?				
Cimex lectularius	p		p				p
Pediculus h. corporis				p	P		
Phlebotomus pappatasii			p				
Psychoda alternata	p						
Pulex irritans		p	p				

P = Physiological resistance
B = Behavioristic resistance
O = Not resistant
Capital letters = Confirmation by experimentation
Lower case = General field observations only

lation increasingly susceptible to a break in the threshold of transmission. It is hoped that an intensified program of fundamental research on all aspects of this problem may provide a satisfactory solution before any such situation develops.

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FIELD INVESTIGATIONS ON THE USE OF HEAVY DOSAGES OF SEVERAL CHLORINATED HYDROCARBONS AS MOSQUITO LARVICIDES[1]

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Field studies with technical DDT and BHC were begun near Savannah, Georgia, in 1948 to develop a mosquito larviciding treatment that would have an extended residual larvicidal action (Mathis and Quarterman, 1949). Studies were expanded in 1949 to include several newer chlorinated hydrocarbon insecticides and preflood treatments as well as residual larvicidal applications[1]. Very limited investigations were continued in 1950 and 1951.

PROCEDURE

These investigations were conducted in small land-locked cypress and blackgum ponds or borrow pits, varying in size from approximately 0.1 to 2 acres. The emergent vegetation ranged from only marginal to a cover of 75 per cent, and most of the ponds were very rich in organic material. These ponds were not subject to strong wind and wave action nor to continuous exchange of water by inflow or outflow except in a few cases which are discussed separately.

A type of selective sampling was employed which was restricted to those portions of each pond that contained vegetation, where past experience had shown that mosquito larvae would be found if any were present in the pond. Samples were taken with dippers uniformly throughout the favorable portions of each pond. Except in the preflood applications, all ponds were sampled before and at weekly intervals after treatment as long as the treatments were considered to be effective. In the preflood tests, the ponds were sampled after flooding at approximately weekly intervals. The mosquito larvae were classified as small, intermediate (2nd and 3rd instars), or large; anopheline or culicine.

In this study, no attempt was made to measure the degree of control by comparison of mosquito larval populations. Conclusions as to the period of effectiveness of each treatment were based on the overall picture of larval development in each pond separately after treatment. A treatment was considered to have become ineffective when any weekly inspection revealed the presence of large larvae, supported by an increase in the stage of development and distribution of the immature larvae present as compared to the previous weekly inspection in that particular pond. In effect, the degree of control obtained was approximately 100 per cent during the period when the test ponds were considered to be under satisfactory control.

Three untreated check ponds were selected for routine inspections which were made concurrently with those in the residual larviciding tests, and three for the

[1] The term "residual larvicidal application" as used in this paper refers to the treatment of ponds containing water and mosquito larvae at the time of treatment.

preflood treatments. The main purpose of the observations in untreated check ponds was to show the general trend of mosquito breeding, especially in connection with the periods when the water level was falling rapidly, or immediately after reflooding. All inspections in the check ponds showed some mosquito breeding, usually with all instars of larvae present, although the population densities varied widely from pond to pond and within the same pond. Large larvae, either culicine or anopheline, were found in over 80 per cent of the inspections made in the check ponds.

Since the preliminary work with heavy dosages of benzene hexachloride in 1948 indicated that this treatment might be applied to ponds without harmful effects to the fish, two borrow pits which had been stocked previously were selected for routine treatments to determine the effect on fish. A third borrow pit was left untreated to serve as a check.

Gross observations were made on the condition of other aquatic organisms at each examination of all test ponds.

EQUIPMENT AND OPERATIONS

The equipment employed was a conventional hand-operated pressure sprayer such as that commonly used in hand larviciding (Ferguson *et al.*, 1947). In the residual larvicidal applications an atomizing nozzle was used, while in the preflood treatments a 5002 Spraying Systems nozzle which produced a flat fan-shaped spray pattern was used. All residual larvicidal treatments were made at the rate of 2 gallons of finished spray per acre, using 30-foot swath widths and a 75-feet-per-minute pace. In the preflood applications 10 gallons of finished spray per acre were used, and the ground surface was sprayed within what was considered to be the boundary of the high water mark.

FORMULATIONS

All formulations were applied as water emulsions containing xylene as solvent and Triton X–155[2] as emulsifier, except that the dieldrin applied in 1949 was prepared from a 25 per cent emulsifiable concentrate supplied by the manufacturer.

The following insecticidal formulations were tested:

1. DDT, tech. grade	1 or 3 pounds
Triton X–155	76 ml.
Xylene	to make 1 gallon
2. DDT, tech. grade	3 pounds
Triton X–155	76 ml.
Rosin	152 grams
Xylene	to make 1 gallon
3. DDD, tech. grade	3 pounds
Triton X–155	76 ml.
Xylene	to make 1 gallon

[2] Rohm and Haas Company, Philadelphia, Pa. This does not represent an endorsement of the product by the Public Health Service.

4. Toxaphene, tech. grade	1 or 3 pounds
Triton X-155	76 ml.
Xylene	to make 1 gallon
5. Benzene hexachloride, tech. grade (12 per cent gamma isomer)	1 pound
Triton X-155	76 ml.
Xylene	to make 1 gallon
6. Dieldrin (commercial formulation)	25 per cent emulsifiable concentrate
7. Dieldrin, tech. grade	1 pound
Triton X-155	76 ml.
Xylene	to make 1 gallon

In ponds which received residual larvicidal treatments 1 gallon of water was added to 1 gallon of each of the concentrates to make a finished emulsion spray of 2 gallons to cover one acre. In the preflood treatments 9 gallons of water were added to 1 gallon of concentrate to make a finished spray of 10 gallons to cover an acre.

Previous work with heavy dosages of DDT (Mathis and Quarterman, 1949) indicated that the toxic agent remained on the vegetation and debris in the treated ponds. Tests with DDT formulations to which pine gum rosin had been added for outdoor residual sprays against adult insects had shown that the addition of rosin prolonged the effectiveness of the material (Jensen *et al.*, 1950). Therefore, tests were made with DDT formulations with and without rosin to determine if the addition of rosin would prolong the larvicidal effectiveness of the DDT.

RESULTS

Residual larvicidal treatments. A summary of the results obtained in these tests is given in Table 1. Most of the ponds in which the residual larvicidal applications were made became dry for various periods after treatment before larvae were found in them. Since the untreated check ponds had larvae in them the first week after reflooding, it was assumed that the treatments were still effective if the treated ponds did not also have larvae in them at this time.

Dieldrin was the most effective of the materials tested. In one pond which was treated in April 1949 at the rate of 1 pound of dieldrin per acre, effective control of mosquito larvae was obtained throughout the 1949 and 1950 seasons. The appearance of immature forms of other insects in October of 1950 indicated that the treatment was becoming ineffective at that time. In two other ponds treated with this dosage in August 1950, control was obtained until October 1951.

DDT at 3 pounds per acre was the second most effective treatment, giving control of mosquito larvae for periods ranging from 10 to over 25 weeks. The addition of rosin as an adhesive failed to increase the residual larvicidal action of the DDT.

The results with technical BHC (12 per cent gamma isomer) at the rate of 1 pound per acre verified the work done in 1948. Effective control of mosquito larvae was obtained for periods ranging from 3 to 8 weeks. In the two ponds in which control was effective for only 3 weeks, heavy rains after treatment greatly

increased the volume of water in both ponds and one of them completely overflowed.

As will be noted from the data in Table 1, the effectiveness of the DDT and BHC treatments was drastically reduced in ponds subject to constant inflow and outflow of water from artesian wells.

Preflood larvicidal treatments. The selection of the larvicides used in these tests was based primarily on their residual qualities as evidenced by their use in other studies. Treatments were applied from May 25 through June 7, 1949, all ponds being dry at the time of treatment. The ponds were filled by rains from June 8–13. The period between the time of treatment and flooding of the ponds

TABLE 1

Summary of results of residual larvicides for the control of mosquito larvae

NUMBER OF PONDS TESTED	INSECTICIDE	DOSAGE IN LBS. PER ACRE	DURATION OF EFFECTIVENESS IN WEEKS	COMMENTS
5	DDT	3	13, 18, 19, 20, 23	Small landlocked ponds
5	DDT and Rosin	3	10, 10, 13, 24†, 25†	Small landlocked ponds
5	BHC (12% gamma)	1	3, 3, 6, 7, 8	Small landlocked ponds
5*	Dieldrin	1	62, 62†, 78	Small landlocked ponds
2	Dieldrin	0.5	11†, 13†	Small landlocked ponds
2	Dieldrin	0.25	8, 12†	Small landlocked ponds
2	Toxaphene	1	4, 6	Small landlocked ponds
2	DDT	3	3, 6	Water from artesian well flowing through pond constantly.
4	BHC (12% gamma)	1	1, 2, 2, 2	Water from artesian well flowing through pond constantly.

* Two ponds treated in 1950 at this dosage maintained control for the remaining 8 weeks of the breeding season and were dry throughout 1951.

† Indicates treatment in pond was still effective when observations discontinued because the pond became dry or because of the onset of cold weather.

varied from slightly less than 1 week to a little over 2 weeks. A summary of the results of these tests is given in Table 2.

In these treatments, also, dieldrin was the most effective material tested, followed by DDT and DDD, which were about equal in effectiveness at 3 pounds per acre. The addition of rosin to the DDT formulation tested again failed to increase the period of residual effectiveness of the treatment.

Species of mosquito larvae. A total of 87 larval collections was made and the following culicine species were identified: *Uranotaenia sapphirina*, *Culex erraticus*, *Culex apicalis*, *Culex pilosus*, *Aedes mitchellae*, *Aedes atlanticus* and *Psorophora columbiae*. The first two species made up a very large part of the culicine collections. Some anopheline larvae were also collected, all of which were identified as *Anopheles crucians*.

Effects of treatments on fish and other aquatic organisms. Gross observations were made in all test ponds on the effects of the larvicides on aquatic organisms other than mosquito larvae. Aquatic insects were greatly reduced by all of the treatments. Fish were practically eliminated by the heavy dosages of all of the materials tested, except the 1-pound-per-acre applications of technical benzene hexachloride. Dieldrin was the most toxic, dead water beetles being found 10 months after the application of 1 pound of this compound per acre. Although adult frogs were killed by the application of 1 pound of dieldrin per acre, tadpoles soon reappeared and apparently were not affected by the residue. Tadpoles also appeared to develop normally in ponds which had received 3 pounds per acre of dieldrin as a preflood treatment. Another organism frequently found alive in the ponds treated with dieldrin was a phyllopod crustacean commonly known as "fairy shrimp".

TABLE 2

Summary of results of preflood treatments for the control of mosquito larvae during 1949

NUMBER OF PONDS TESTED	INSECTICIDE	DOSAGE IN LBS. PER ACRE	DURATION OF EFFECTIVENESS IN WEEKS	COMMENTS
3	DDT	1	3, 4, 4	Small landlocked ponds
3	DDT	3	5, 17*, 17*	Small landlocked ponds
3	DDT and Rosin	3	9, 11, 17*	Small landlocked ponds
3	Dieldrin	3	18*, 18*, 18*	Small landlocked ponds
3	Toxaphene	3	4, 4, 5*	Small landlocked ponds
2	DDD	3	14, 17*	Small landlocked ponds

* Indicates treatment in pond was still effective when observations discontinued because the pond became dry or because of the onset of cold weather.

Tadpoles of the following species were collected from ponds that had been treated with dieldrin at 3 pounds per acre as preflood treatments: *Microhyla carolinensis*, *Hyla cinerea*, *Hyla femoralis*, *Hyla gratiosa*, *Hyla squirella*, and *Pseudacris* spp.

Studies in 1948 indicated that technical benzene hexachloride (12 per cent gamma isomer), when applied as an emulsion at the rate of 1 pound of BHC per acre, was not immediately harmful to fish. To investigate this problem more fully, 3 ponds were stocked with fish in 1949, 2 of which were treated routinely at intervals of 5 weeks with BHC emulsions at the rate of 1 pound of technical BHC per acre per application. The 3rd served as an untreated check. Five treatments were made each year on both of the 2 treated ponds, between May and September in 1949, 1950, and 1951. All 3 ponds received 7 applications per year of a 5–10–25 commercial fertilizer at the rate of 100 pounds per acre, applied at approximately 6-week intervals beginning in February, 1949. This fertilizer was applied to increase the food for fish during the period studied.

After larvicidal treatments were completed each year, the ponds were seined to determine the fish populations, and after the fish were identified and counted they were returned to the ponds for further studies. Table 3 gives the fish popu-

lations of the 3 ponds before treatments were begun in the spring of 1949 and after they were completed in the fall of 1951.

This study was designed only to determine if routine treatments with BHC would have any drastic effect on fish populations when used at the approximate intervals necessary to control mosquito larvae continuously. The two most important species, black bass and blue gill bream, increased in numbers considerably in treated as well as untreated ponds. Bullhead catfish and suckers were also present in one treated pond and in the check pond throughout the study, but since many of each were removed at each sampling the numbers are not included. The treatments apparently had no more effect on these species than they did on the others present. It will be noted that the orange spot sunfish declined or dis-

TABLE 3

Fish populations in test ponds before and after five treatments per year with technical benzene hexachloride (12 per cent gamma isomer) applied for three consecutive years at 1 pound per acre per application

TYPE OF FISH	UNTREATED POND		TREATED PONDS			
			Pond No. 1		Pond No. 2	
	March 1949	October 1951	Before treat. 1949	After treat. 1951	Before treat. 1949	After treat. 1951
Black bass	13	137	8	56	8	16
Warmouth bass	9	21	26	5	9	30
Orange spot sunfish	100	0	175	0	195	28
Blue gill bream	34	5860	329	1140	10	2500
Golden shiner	5	5	44	16	155	30
Total fish	161	6023	582	1217	377	2604
Area in acres	0.53		0.47		0.22	
Max. depth of water	5.5 ft.		4.5 ft.		4.0 ft.	

appeared in all ponds and the golden shiners apparently did not thrive in any pond, but these reductions cannot be attributed to the treatments since the check followed a similar trend. The warmouth bass also followed a somewhat similar pattern.

Samples of black bass, warmouth bass, blue gill bream, suckers and catfish were removed annually from the treated ponds and submitted for taste tests to several different individuals to determine if the benzene hexachloride had affected the taste of the fish. No off-taste was reported for any of the fish tested during the first two years. An off-taste was reported in a black bass at the end of the third year, but this may have been caused by the effects of the unusually low water level of the pond prevailing during that summer.

SUMMARY

Several of the newer chlorinated hydrocarbons were tested near Savannah, Georgia, at heavy dosages in an effort to establish a long period of effective con-

trol against mosquito larvae. In residual larvicidal tests in small landlocked ponds effective control for differing periods was obtained with heavy dosages of DDT, benzene hexachloride, toxaphene and dieldrin. Dieldrin was the most effective and at one pound per acre controlled breeding for 62–78 weeks. DDT at 3 pounds per acre was effective for only 13–23 weeks, and the addition of rosin did not significantly improve its action. The other two toxicants gave less enduring results. The effectiveness of DDT and BHC was greatly reduced by a constant flow of water into or through test ponds.

Preflood applications, at 3 pounds per acre to dry ponds subsequently filled by rains, also showed the superiority of dieldrin, which controlled breeding for more than 18 weeks, closely followed by DDT and DDD which in some instances exhibited a residual action for more than 17 weeks.

Five treatments per year with technical benzene hexachloride applied each summer for 3 consecutive years at the rate of 1 pound per acre to the ponds resulted in no fish injury which could be attributed to the effects of the BHC treatments. All other materials used in these tests were decidedly harmful to fish and other aquatic organisms and should never be applied in the above concentrations where these are of importance.

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QUANTITATIVE FIELD STUDIES ON A CARBON DIOXIDE CHEMOTROPISM OF MOSQUITOES[1]

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The host preferences of different species of mosquitoes, which are relevant to the epidemiology of diseases carried by these vectors, have long been recognized and long unexplained. A previous study (Reeves, 1951) confirmed that various mosquito species were attracted to carbon dioxide gas. This finding suggested that a carbon dioxide chemotropism may be a major factor in host selection. Investigations of the role of mosquitoes in the epidemiology of the arthropod-borne viral encephalitides in Kern County, California in 1951 showed the practicability of studies on the quantity of carbon dioxide preferred by different species of mosquitoes. A more extensive, more precise study was therefore undertaken and forms the basis of the present report.

In general the procedure was to provide concentrations of carbon dioxide representing approximately the amount excreted by the chicken, man and the horse (or cow) and then to find out the number and species of mosquitoes which found these baits attractive.

METHODS AND MATERIALS

Two stable traps of the design described by Bates (1944) were the standard apparatus for mosquito collection. Tanks of compressed carbon dioxide of 25 pound (6,000 liter) capacity were the source of gas. The volume released was controlled by a gas pressure regulator with a gauge dial calibrated in pounds per square inch. The final release was through the orifice of a glass capillary tube with the tip encased in heavy rubber tubing to protect it from breakage. The glass capillary tubes were prepared and calibrated by measuring the flow of carbon dioxide in milliliters per minute which passed through a wet gas meter at various differential pressures. Four such tubes were used: two covered the range of flow of 20 to 300 ml. per minute, and two the range of 600 to 2,500 ml. per minute.

Three rates of release were selected for these studies on the basis of various published estimates: 25 ml. (chicken), 250 ml. (man), and 2,500 ml. (horse or cow) per minute. Because of the lack of sensitivity of the pressure gauge it was not possible to maintain the flow precisely at these levels for 12 hours or longer. Therefore, at the beginning of a trial the apparatus was set to release slightly more than the desired amount and by the termination of the trial the amount being released had decreased to a level below that selected. Readings were taken

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at the beginning and end of each trial, and the range and average flow were computed. The mean actual flow is presented in the tables; it was usually below the selected level. In the discussion of the results, in order to simplify the discussion, the three levels—25 ml., 250 ml. and 2,500 ml.—will be used to represent the ranges studied. All trials in which the equipment failed to function satisfactorily were excluded, regardless of the number of mosquitoes collected.

On each test two traps spaced approximately 40 feet apart were run simultaneously. When the attractiveness of two flows was being compared, the different flows were alternated between the two traps on successive nights. This decreased the chance that average collection figures would be affected by location of the traps, rather than by differences in the attractiveness of gas concentrations.

The 250 ml. flow was selected as the constant one; it was compared with 25 ml. and 2,500 ml. The latter two were never compared directly.

Tests were made in two areas: The first was on the banks of a natural watercourse known as Poso Creek. Conditions in this stream were favorable for development of aquatic stages of *Culex tarsalis* and large numbers were present along its margins throughout the study. As a result, the population of *C. tarsalis* adults was very large. Only small numbers of other species inhabited the area. The traps were placed in the shade of trees on the creek bank within 50 feet of breeding areas and adult resting shelters. The second area, Palm Ranch, was a large ranch on the margin of an arid region. A number of different species of mosquitoes were to be found in the area, probably because the water sources, which resulted from irrigation, were of diverse types. The three principal species were *C. tarsalis*, *Culex quinquefasciatus* and *Aedes nigromaculis*. In this area the traps were placed in an open unshaded pasture on which several hundred cattle were feeding.

Trials were made on 20 nights. Most of them were begun between 5 and 6 P.M. when the gas was turned on and the flow regulated. They were terminated between 8 and 9 A.M. the following morning when the gas was turned off and all mosquitoes in the traps were collected in cages and taken to the laboratory for identification and counting.

At Palm Ranch, because of its distance from the field station and time taken to get there, in trials at the two lower levels (25 ml. and 250 ml.) the gas was allowed to run continuously day and night. Mosquitoes were removed each morning between 9 and 10 A.M. and at the same time cylinders, with gauges and capillary tubes attached and running, were interchanged between traps. In trials with 2,500 ml. it was necessary to limit runs to 16 hour periods or less because of the short life of cylinders at this flow (only 3 runs).

RESULTS

Because of the difference in the size and species of the mosquito populations in the two study areas, each area is considered separately. All collection figures refer only to female mosquitoes; only 22 males (all *C. tarsalis*) were collected.

Poso Creek. A total of 5,443 mosquitoes was collected in this area in 18 trials

(Table 1). *C. tarsalis* was the only species trapped in numbers adequate to permit comparison of the attractiveness of the different concentrations of carbon dioxide. Progressively larger numbers of this species were attracted as the rate of flow of carbon dioxide was increased; even the smallest amount attracted large numbers.

TABLE 1

Species and number of female mosquitoes collected in trials with different flows of carbon dioxide, Poso Creek area

MEAN FLOW OF CO_2 PER MINUTE	NO. OF TRIALS	*C. tarsalis*		*C. quinquefasciatus* TOTAL	*C. thriambus* TOTAL	*A. nigromaculis* TOTAL	*A. franciscanus* TOTAL	ALL SPECIES	
		Total	Mean per trial					Total	Mean per trial
24 ml.	5	863	173	4	1	0	0	868	174
241 ml.	11	3,069	279	25	1	5	0	3,100	282
2475 ml.	2	1,453	727	1	0	8	13	1,475	738

TABLE 2

Species and number of female mosquitoes collected in trials comparing the attractiveness of two different flows of carbon dioxide on the same night, Poso Creek area

MEAN FLOWS OF CO_2 PER MINUTE COMPARED	NO. OF TRIALS	MOSQUITOES COLLECTED		
		C. tarsalis		Total other species
		Total	Mean per trial	
26 ml.	4	665	166	3
224 ml.		980	245	13
235 ml.	2	332	166	6
2500 ml.		1,453	727	26

TABLE 3

Species and number of female mosquitoes collected in trials with different flows of carbon dioxide, Palm Ranch area

MEAN FLOW OF CO_2 PER MINUTE	NO. OF TRIALS	*C. tarsalis*		*C. quinquefasciatus*		*A. nigromaculis*		*A. dorsalis* TOTAL	ALL SPECIES	
		Total	Mean per trial	Total	Mean per trial	Total	Mean per trial		Total	Mean per trial
32 ml.	3	280	93	77	26	5	2	0	362	121
242 ml.	8	1,110	139	174	22	140	18	9	1,433	179
2425 ml.	4	664	166	49	12	301	75	12	1,026	257

Consideration of the specific tests in which the effects of two different rates of flow were successfully compared on the same night shows that 250 ml. attracted large numbers when it was competing with 25 ml., but much smaller numbers when competing with 2,500 ml. (Table 2).

Palm Ranch. A total of 2,821 mosquitoes were collected in this area in 15 trials (Table 3). *C. tarsalis*, *C. quinquefasciatus* and *A. nigromaculis* were collected in numbers sufficient to allow analysis. The number of *C. tarsalis* again increased

as the flow of carbon dioxide was increased. The trend was quite similar to that at Poso Creek, even though the total population of this species at Palm Ranch appeared to be much less. At the higher rates of carbon dioxide release the number of *C. quinquefasciatus* collected decreased. *A nigromaculis* and possibly *Aedes dorsalis* were attracted in the largest numbers to the highest rate of release; practically none were collected at the 25 ml. level.

In specific comparative trials (Table 4), the number of *C. tarsalis* collected in 250 ml. tests was higher when this bait competed with the 25 ml. rate than when it competed with the 2,500 ml. rate. The same may be true for *C. quinquefasciatus*, but not for the two species of *Aedes* collected.

TABLE 4

Species and number of female mosquitoes collected in trials comparing the attractiveness of two different flows of carbon dioxide on the same night, Palm Ranch area

MEAN FLOWS OF CO_2 PER MINUTE COMPARED	NO. OF TRIALS	MOSQUITOES COLLECTED						
		C. tarsalis		*C. quinquefasciatus*		*A. nigromaculis*		*A. dorsalis*
		Total	Mean per trial	Total	Mean per trial	Total	Mean per trial	Total
32 ml.	3	280	93	77	29	5	2	0
250 ml.		459	153	104	35	6	2	1
238 ml.	4	498	125	56	14	54	14	7
2400 ml.		664	166	49	12	301	75	12

DISCUSSION

The attractiveness of different environmental concentrations of carbon dioxide to different species of mosquitoes may now be said with assurance to have biological implications. Present data, although limited, reveal differences of a magnitude justifying more extensive study. The tendency of the number of attracted *A. nigromaculis* to increase when the concentration of carbon dioxide is higher, in contrast to the tendency of the numbers of *C. quinquefasciatus* to decrease (Figure 1), fits the field observations on their host preferences. It is possible that *A. nigromaculis* is not attracted to birds because this mosquito may not be able to detect the relatively small amounts of carbon dioxide released by chickens and smaller birds, and finds them only occasionally and by accident in a random search for hosts. As concentrations of the stimulant are increased, as though by larger hosts, the range of detection is entered and the hosts are easily found; the optimum range may be the amounts of this gas given off by larger animals.

In contrast are the reactions of *C. quinquefasciatus* and *C. tarsalis* (Figure 1). The relatively lower concentration of carbon dioxide given off by avian hosts may have the maximal attractiveness for the first of these two species, which apparently is attracted less as concentrations increase. A pattern of this type would be expected of an aviphilic species.

C. tarsalis reacted differently than was expected; large numbers were attracted to all three levels of carbon dioxide and the numbers collected increased as the level increased. Former observations had indicated that this mosquito is mainly

aviphilic, feeding most frequently on birds, but it is also known to feed commonly on large mammals. If this species is equally or more attracted to the greater concentrations of carbon dioxide, one might question its aviphilic tendencies or the specificity of this stimulant. However, further consideration of environmental factors may resolve this dilemma. In the areas where the host preference tendencies of *C. tarsalis* have been discerned, the populations of wild and domestic birds have been very large. Avian hosts far outnumbered large mammals and were therefore more available. Under these circumstances, in a search for blood,

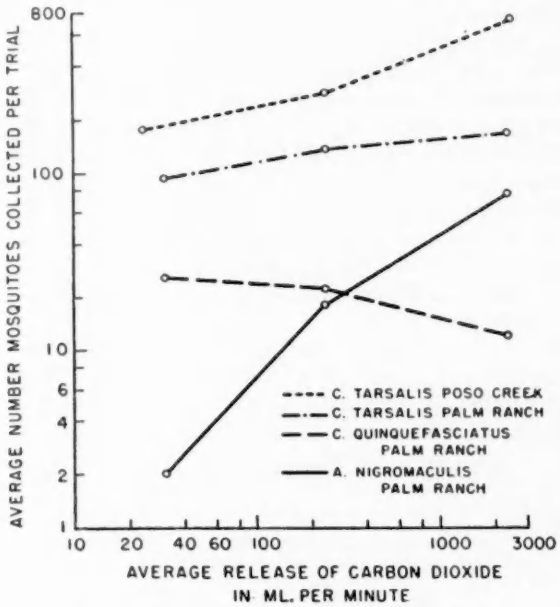

FIG. 1. Average number of mosquitoes attracted by different flows of carbon dioxide.

a species of mosquito capable of detecting low concentrations would have a much better chance of finding an avian source than a large mammalian source of blood. This would be particularly true if their range of detection is short.

On entering traps to remove mosquitoes, the collector was attacked quickly unless mosquito repellent was used. *C. tarsalis* seldom bites in the day time, but under the conditions of these experiments it seemed unduly stimulated. It was most common to observe mosquitoes probing at the rubber tubing where gas was released as if they were in search of blood.

The present data are too limited to permit more than an observation of trends and the drawing of inferences. Additional studies are needed in a greater variety of environmental situations, particularly with different sizes and species of mosquito populations and with other amounts of carbon dioxide.

The phenomenon of interference of one level of stimulant with another is of

interest because of its possible role in ecological situations where more than one host species is present and is therefore possibly in competition with other hosts in the attraction of mosquitoes. The number of *C. tarsalis* attracted when the rate was 250 ml. increased in both study areas after the alternate rate of 2,500 ml. was replaced by 25 ml. (Tables 2 and 4). This phenomenon could be explained either by competition between the two attractants or an increase in mosquito population at this time. One might question whether the mosquito population would increase coincidentally in two widely separated areas but, of course, it is a possibility. (Competition of hosts is reminiscent of the efforts to employ large domestic animals as a protective barrier for man in malaria control.)

It cannot be concluded that carbon dioxide is more attractive to *C. tarsalis* than to the other species collected because the population of this species in the two areas was probably larger. *C. tarsalis* is however, the one species sufficiently common in both areas to permit comparison of the attractant. Figure 1 illustrates that trends in the number of specimens attracted at the two lower rates of carbon dioxide release ran parallel in spite of the differences in the size of the mosquito population in the two areas. The relationship diverges at the higher rate of stimulant release, although in both areas the number of mosquitoes was higher than at the lower rates tested. This divergence could well be due to factors such as population pressure, competition of other attractants or variability in data due to the small number of tests run.

Development of a technique for sampling the population of mosquitoes preferring a certain host may be possible by using the principle of releasing a quantity of carbon dioxide comparable to that given off by the host in question. In investigations of mosquitoes suspected as disease vectors, such a sampling technique could be of considerable epidemiological value. The recent Canadian studies (Brown, 1951) of factors attracting *Aedes* mosquitoes to man included this possibility by releasing from robots carbon dioxide in amounts approximating that released by man. The findings of this study appear to be consistent with those of the present investigation.

SUMMARY

Carbon dioxide, released at rates of approximately 25 ml., 250 ml. and 2,500 ml. per minute, was successfully employed as a host-simulating mosquito attractant. These rates were selected because they represent approximately the average amounts of carbon dioxide given off by chicken, man, and horse or cow, respectively.

Large numbers of female mosquitoes of several species were attracted into traps baited with these concentrations of carbon dioxide. The various species of mosquitoes responded differently. *Aedes nigromaculis* was attracted in the greatest numbers to the higher concentrations. More *Culex quinquefasciatus* were attracted to the least amount. *Culex tarsalis* was greatly attracted to all three concentrations, but the largest numbers were attracted to the traps where the rate of release was highest. These observations are correlated with available knowledge of the host preference habits of these species and they suggest a direct interrelationship of host-seeking and carbon dioxide chemotropism.

These observations are of potential value in epidemiological studies of mosquito-borne diseases and offer a new investigative tool in such research.

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THE RELATIONSHIP OF EXPOSURE TIME TO THE MOLLUSCICIDAL ACTIVITY OF COPPER SULFATE[1]

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Recent publications on the control of bilharziasis (Abdel Azim, 1948; Wright, 1950; McMullen *et al.* 1951) have emphasized the importance of developing more effective molluscicides for use against the snail hosts of this widespread disease. The most common approach to the molluscicide development problem is one in which a large number of chemical compounds are screened in the laboratory and the most promising ones subsequently tested in the field. Another and perhaps not less important method of attack involves a study of the manner in which environmental factors (temperature, contact time, type of water, etc.) influence molluscicide activity.

In discussing the requirements of an ideal molluscicide, Abdel Azim (1948) states that the substance should be sufficiently active to kill snails within 24 hours, and this somewhat arbitrary exposure period has been used by a number of workers interested in evaluating compounds for their molluscicidal activity (Kuntz *et al.*, 1950; Batte *et al.*, 1951; Barry *et al.*, 1950; Hoffman *et al.*, 1952). However, no detailed study of the relationship between exposure time and molluscicide activity has been reported by workers in this field. The present communication is concerned with the interaction of these two variables in the case of copper sulfate.

Khalil (1932) reported an extensive series of experiments with copper sulfate in distilled, tap, Nile, and pond water using *Bulinus*, *Planorbis*, and *Physa* as the test animals. Exposure times of from 1 to 8 days were employed. Unfortunately the choice of copper sulfate concentration in 320 of the 496 tests was such that all snails were killed. Khalil concluded that *Planorbis* was more resistant to copper sulfate than either *Bulinus* or *Physa* and that the effect of copper sulfate on the snails increased in proportion to the exposure time.

Kuntz and Wells (1951) exposed *Biomphalaria* and *Bulinus* for periods of 3, 6 and 24 hours to copper sulfate (6, 12 and 20 p. p. m.), to 2,4-dinitro-6-cyclohexylphenol (3 p.p.m.) and to the dicyclohexylamine salt of the latter compound (3 p.p.m.). The molluscicide solutions in tap water were in contact with canal mud and aquatic plants at room temperature. In most cases the dinitro compounds at 3 p.p.m. appeared to be superior to copper sulfate at 20 p.p.m. Percentage mortalities, however, showed wide variations, which the authors attributed to differences in susceptibility among the large number of snails used.

In a study of the effect of copper sulfate in standing tap water, Nolan (1950) found that *Australorbis glabratus* and *Biomphalaria boissyi* were more resistant

[1] The opinions or assertions contained in this article are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department.

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to copper sulfate at 20 p.p.m. than were *Bulinus contortus*. In all three species percentage mortality increased with exposure time, although somewhat irregularly in the case of *B. boissyi*. Nolan reported mortalities of 55 per cent (*A. glabratus*); 58 per cent (*B. boissyi*); and 83 per cent (*B. contortus*) following one hour's contact with 20 p.p.m. copper sulfate.

In the present investigation exposure time was related to LD_{50} rather than to percentage mortality since the reliability of toxicity estimates based on quantal response data is greatest at LD_{50} and becomes progressively less as 0 and 100 per cent effects are approached (Gaddum, 1933).

MATERIALS AND METHODS

Exposure tests were carried out in a constant temperature bath at 20°C. using a series of one-liter beakers. Each beaker contained 16 snails in a solution prepared by diluting concentrated $CuSO_4 \cdot 5H_2O$ with filtered pond water to a final volume of 800 cc. Thirty-two adult snails were tested at each copper sulfate concentration.

Following the exposure period, the snails were rinsed well, transferred to fresh pond water containing *Elodea* and returned to the constant temperature bath for one day. The living and dead snails were then placed in separate beakers containing fresh pond water and *Elodea*. This procedure was repeated the following two days making a total post-exposure period of approximately 72 hours at 20°C. Percentage mortality was then determined.

Snails for the exposure tests were kept in a series of large (700 to 1500 liter) shaded, outdoor ponds previously filled with water from an irrigation drain near Cairo and stocked with adult *B. boissyi* taken from the same drain during May and June, 1952. The ponds were supplied with copious quantities of *Elodea* which served as a source of both oxygen and food for the snails. Water from the snail ponds was yellow in color and varied in pH from 7.9 to 9.3 (av. = 8.5); readings taken late in the afternoon were approximately half a pH unit higher than those taken early in the morning due, in all probability, to the increased metabolic activity of the *Elodea* during the day.

DISCUSSION OF RESULTS

The effect of copper sulfate on *B. boissyi* following exposures ranging from 6 to 72 hours is shown in Table 1. LD_{50} values (Table 2) were estimated graphically by plotting the percentage mortality data (Table 1) against the corresponding copper sulfate concentrations using logarithmic probability paper (Litchfield *et al.*, 1949).

With the exception of the value corresponding to the 24-hour exposure period, log LD_{50} plotted against the logarithm of the exposure time closely approaches a linear relationship. LD_{50} plotted directly against exposure time (Fig. 1) emphasizes the rapid decrease in the amount of copper sulfate required to kill *B. boissyi* as the time of exposure is increased, particularly within the first 24 hours.

It is of interest to compare the mortality data of Table 1 with that which other workers have reported. Nolan (1950) found that 78 per cent of her *B. boissyi*

were killed by a 2-hour contact with copper sulfate at 20 p.p.m., while Kuntz and Wells (1951) reported a 41 per cent (average) kill for 6 hours at this concentration. A logarithmic probability graph of the present data indicates a 46 per cent kill for 12-hours contact with 20 p.p.m. copper sulfate. Local Egyptian *B. boissyi* are either much more hardy than those which Nolan tested or (more probably) the conditions of her experiment were less favorable for snail survival than those used in the present investigation.

Preliminary data on the rate at which various copper sulfate concentrations in tap and pond water decrease with time are shown in Table 3. These data indicate that copper sulfate is removed *less* rapidly from pond water than from

TABLE 1

Mortality of B. boissyi *following exposure to copper sulfate in pond water at 20°C.**

EXPOSURE (HOURS)	$CuSO_4 \cdot 5H_2O$ (p.p.m.)	MORTALITY† (%)	EXPOSURE (%)	$CuSO_4 \cdot 5H_2O$ (p.p.m.)	MORTALITY† (%)
6	24	0	24	2.0	0
	40	6		3.2	19
	67	81		5.1	91
12	9	0	48‡	0.7	0
	18	34		1.4	19
	36	94		2.8	81
18	8	22	72‡	1.0	3
	12	72		1.3	38
	18	94		1.6	81

* These tests were carried out between 12 May and 6 July 1952.

† Percentage mortality following a 72-hour post-exposure period. No deaths occurred among 80 control snails.

‡ Solutions replaced by fresh copper sulfate in pond water at 24-hour intervals.

TABLE 2

LD_{50} values for B. biossyi *exposed to copper sulfate in pond water at 20°C.*

EXPOSURE (HOURS)	6	12	18	24	48	72
LD_{50} (ppm. $CuSO_4 \cdot 5H_2O$)	55	21	10	3.8	2.0	1.35

tap water at 20°C. The lower mortality rates characteristic of the present experiments in natural waters are therefore not due to the presence of factors which rapidly remove copper sulfate from solution.

The temperature at which the present study was carried out (20°C.) is somewhat lower than that reported by Nolan (25–26°C.) and within the range of temperatures used by Kuntz and Wells (19–25°C.) but it is unlikely that this variation in itself would account for the difference in results. The factors responsible for these variations remain to be investigated, but it would appear that tests carried out in tap water are apt to be misleading in regard to the molluscicidal activity of copper sulfate in natural waters favorable for snail colonization.

With regard to the practical application of copper sulfate as a molluscicide

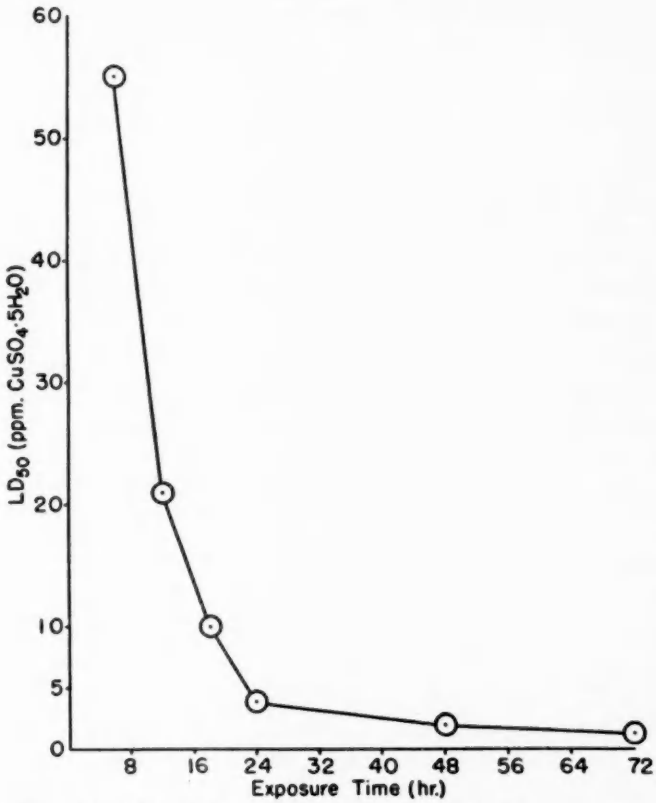

FIG. 1. LD_{50} (*B. boissyi* vs. copper sulfate) as a function of exposure time

TABLE 3

*Copper sulfate concentration changes during contact with tap and pond water**

	$CuSO_4 \cdot 5H_2O$ (PPM.)			
	Initially	After 6 hours	Initially	After 24 hours
Tap water (pH = 8.0)	39.5	29.4	7.1	4.9
	66.0	33.6	4.4	3.6
Pond water (pH = 8.7)	37.9	37.2	6.1	5.4
	63.2	61.4	3.7	3.4

* At 20°C. in the presence of 16 *B. boissyi* in 800 cc. of copper sulfate solution. Copper was determined colorimetrically according to the method of Sandell, "Colorimetric Determination of Traces of Metals", 224 (1944, Interscience Publishers, New York).

the present data underline the importance of maintaining a given copper sulfate concentration for as long a time as possible and suggest that the use of low copper sulfate concentrations maintained for an extended period of time may be more effective and less expensive than the high concentrations which result when solid copper sulfate is dissolved directly in canal water.

SUMMARY

In a study of the relationship between molluscicidal activity and exposure time, *Biomphalaria boissyi* were exposed to copper sulfate in pond water at 20°C. for various lengths of time. Expressed in terms of $CuSO_4 \cdot 5H_2O$, LD_{50} decreased from 55 p.p.m. following a 6-hour exposure period to 1.35 p.p.m. after an exposure of 72 hours. From a comparison of these data with the results of other workers it is concluded that the use of tap water in copper sulfate evaluation tests results in a considerably higher mortality than the use of natural waters from sites favorable for snail colonization. This effect is not due to the presence in natural water of factors which rapidly remove copper sulfate from solution.

It is suggested that the use of low copper concentrations maintained for an extended period of time might be more effective and less expensive than the high concentrations which result when solid copper sulfate is dissolved directly in canal water.

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NOTES ON THE CAT FLEA IN PUERTO RICO

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The recovery of murine typhus from naturally infected cat fleas, *Ctenocephalides felis* (Bouché) by Irons *et al.* (1944) and the suspicion that infection was transmitted to man through their agency invests the cat flea with unusual medical importance. As in the case of all suspected vectors of human disease, epidemiological knowledge can only increase when the determination to species is unequivocal. The literature, particularly that dealing with rat-ectoparasite surveys, shows that this has not been the case in the past for it is replete not only with various circumlocutions in reporting cat and dog fleas but also with erroneous identifications. The probabilities are that the dog flea, *C. canis* (Curtis) is much less common in America than the literature indicates and in fact occurs not at all in a number of localities from which it has been reported, *C. felis* being involved. In the following pages evidence is brought forward to show that one such place is Puerto Rico, and the opportunity is taken to emphasize the characters which separate the two species and record some of the intraspecific variations in the cat flea.

The belief that the records of *C. canis* in Puerto Rico properly belong to *C. felis* is based on the data from recent ectoparasite surveys of rats and dogs and on a re-examination of the published reports. The rat-ectoparasite survey was made in the residential section of San Juan, P.R. known as Santurce from January 17, 1946 to March 8, 1949 wherein a total of 938 (809 *Rattus norvegicus* and 129 *R. rattus*) were collected; and of these, 16 rats, all *R. norvegicus*, were infested with 17 specimens of *C. felis*. (In 1946 there were 12 rats infested with 12 specimens taken during each month except January, July, September and October; in 1947 only one rat taken in January was infested with one specimen; in 1948 three rats were found infested, one with two specimens in April and two with one specimen each in May). The infestation per cent was therefore 1.7 and the mean 0.018. The data do not indicate a seasonal variation but do show a host preference in favor of *R. norvegicus*.

The dog-ectoparasite survey was much less thorough, the number of individuals examined being few; however the data obtained indicate that greater numbers would not have changed the conclusions. Fifteen stray young dogs from the residential section of San Juan were studied in July and August of 1950. They were found to be 100% infested with *C. felis*, more than 200 specimens being taken from them. They were so heavily infested it was not deemed worth-while to derive the exact mean, even if possible to do so.

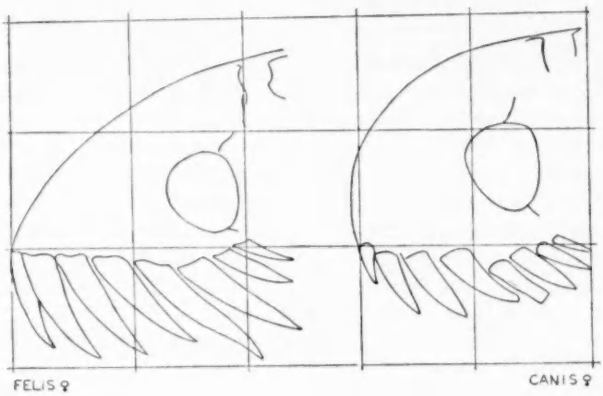
Since the dog flea, *C. canis*, had not been taken in these surveys from either rats or dogs, a re-examination of the published records of that species in Puerto Rico was indicated. In the literature there are a number of records of the dog flea in Puerto Rico. The first to report it from rats was Creel (1913) who found it in various cities of the Island. He did not mention *C. felis* at all and the writer sur-

mises that those who determined his material subscribed to the opinion prevalent among certain specialists then that *C. felis* was a synonym of *C. canis*. The dog flea was again reported from rats in the course of a series of surveys made from 1926 to 1929 at San Juan. In a preliminary report on this work the flea is referred to as "*Ctenocephalus canis*" (Cox, Carrión and Fox, 1928), but in subsequent articles "*Ctenocephalus canis* or *felis*" is used in such a way as to signify doubt in regard to the determination (Carrión, 1932). During this time and afterwards *C. canis* was reported as a pest in houses, on dogs and on mongooses by experiment station workers (Wolcott, 1924 and 1936). Often the determinations of fleas obtained in rat surveys and experiment station work are checked by specialists in the U. S. Department of Agriculture; accordingly word was sent there in the hope that the material which provided the basis for these records might be extant and could be re-examined. The reply received (Muesebeck, 1952) was in part as follows: "The U. S. National Museum collection of *C. canis* has only a single slide from Puerto Rico. According to the label Ewing identified the female on this slide as *C. felis* and the male as *C. canis*. In my opinion both specimens are very definitely *felis* and I am placing them in the collection of *felis*. We have a number of Puerto Rican specimens determined as *C. felis* by Ewing and, in my judgment, correctly so. These are mostly from Mayagüez and were taken from dogs, rats, goats and calves. Included is one specimen (a male), collected by C. W. Hooker in 1912 from a rat, which may, I suppose, be part of the series you mentioned as having been reported as *C. canis*. It is certainly *felis*."

Mention should be made of a recent article on the fleas of Puerto Rico in which *C. canis* is illustrated (Maldonado Capriles, 1945). In reply to a query Prof. Maldonado stated that he could not find any specimens of *C. canis* in Puerto Rico and for his illustration he used an exotic specimen loaned by the U. S. National Museum; he went on to say that he had later examined fleas from dogs in Mayagüez, P.R. and invariably they were the cat flea. Venezuela may possibly be another country where the dog flea does not occur in spite of the records, a circumstance implied by Hecht (1942) who found *C. felis* on rats, dogs and man, but not *C. canis*.

Opinion is unanimous among present day specialists that the cat flea and the dog flea are distinct species. The salient characters which separate them were pointed out years ago and have long been common knowledge: the cat flea has a longer and more pointed head, its first genal spine is longer and the distal portion of the manubrium is not greatly expanded. Rothschild (1905) stressed as well the larger abdominal spiracles of *C. canis* and its different hind tibial chaetotaxy; this last character has been emphasized by Jordan (1943) who wrote, "in *canis* the hind tibia bears two stout bristles dorsally between the apical bristles and the post-median ones, whereas in *felis* the upper of the two is replaced by a small hair." These five important characters were studied in detail in 50 females and 40 males of *C. felis* from the San Juan dogs with the object of ascertaining the intraspecific variations. The specimens of *C. felis* were compared with a male and a female specimen of *C. canis* taken from the silver fox at Ames, Iowa by E. F. Knipling in 1934 and the results were as follows:

Head. The most important character of use in separating the cat flea from the



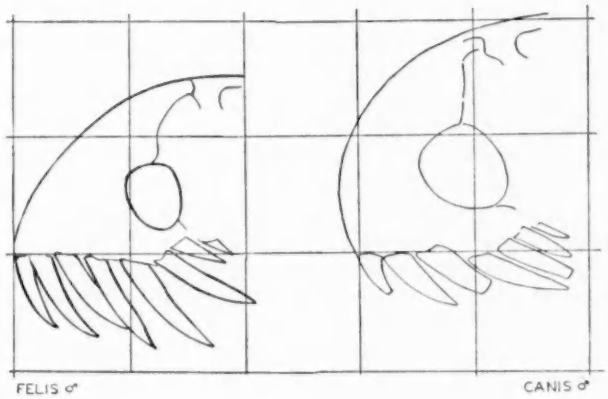


FIG 1

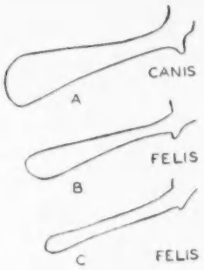


FIG 2

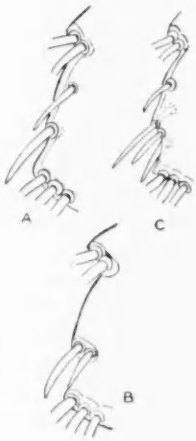


FIG 3 CANIS

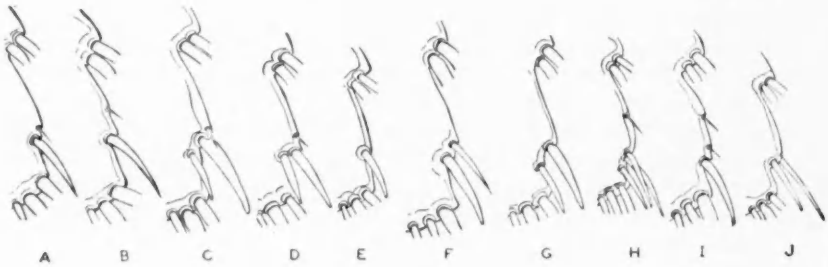


FIG 4 FELIS

FIG. 1. *Ctenocephalides felis* (Bouché) and *C. canis* (Curtis), anterior portion of the heads outlined on grids to show the angle with which the frons meets the genal comb. FIG. 2. A, *C. canis*, manubrium; B, *C. felis*, moderately expanded manubrium; C, *C. felis*, slightly expanded manubrium. FIG. 3. *C. canis*; A and B, dorsal subapical bristles on the hind tibiae of a female; C, the same of a male. FIG. 4. *C. felis*, types of dorsal subapical bristles on the hind tibiae; A–G, females; H–J, males.

dog flea is the shape of the head. Excellent illustrations of the heads may be found in Holland (1949) and Hubbard (1947), and the latter author rightly emphasizes the angle of the frons as the decisive factor. This may be brought out more vividly by outlining the frons and gena on a grid as shown in Fig. 1, where the more acute angle with which the frons meets the genal comb in *C. felis* is made obvious. (Incidentally, if such a grid is drawn over the head figured by C. Fox in 1925 on page 135 of his textbook, it becomes apparent that he had in fact *C. felis* and not *C. canis* as the legend states). The shape of the head in all the Puerto Rican specimens of *C. felis* showed little if any variation and by this character alone the species could be readily identified.

Genal spine. It is very difficult to measure the relative lengths of the first and second genal spines because of their obscure bases and strong curvature; besides they are often not inserted at the same level. The first and second genal spines of the Iowa male specimen of *C. canis* measured in length respectively 56 and 95 (these and all measurements which follow are in microns). The 40 Puerto Rican male specimens of *C. felis* varied greatly as regards the relative lengths of the first and second genal spines; 23 were in the proportion $70 \pm 8 : 92 \pm 9$ and 17 were in $81 \pm 8 : 106 \pm 8$, and the difference between the lengths of the first and second genal spines varied from 11 to 31 distributed as follows: three specimens, 11; three specimens, 14; seven specimens, 17; five specimens, 20; five specimens, 22; nine specimens, 25; four specimens, 28; and four specimens, 31. The first and second genal spines of the Iowa female specimen of *C. canis* measured 56 and 87 respectively. The same genal spines in 50 Puerto Rican female specimens of *C. felis* were highly variable in length; 29 specimens were in the following proportion, $101 \pm 8 : 120 \pm 8$; 12 in $78 \pm 8 : 101 \pm 8$; and nine in $112 \pm 6 : 140 \pm 6$, and the difference between the lengths of the first and second genal spines varied from 11 to 31 distributed as follows: two specimens, 11; nine specimens, 14; eight specimens, 17; eight specimens, 20; six specimens, 22; five specimens 25; nine specimens, 28; and three specimens, 31. The genal comb as a whole varied in the total number of spines as well as in their sizes and relative positions. Of the 40 male specimens of *C. felis*, 32 had on one side the usual complement of eight spines, but three had nine spines and five had seven spines; of the 50 female specimens 40 had eight spines, three had nine spines and seven had seven spines. From the foregoing it will be seen that a number of texts err in their accounts of the genal comb of *C. felis* in stating that there are seven or eight spines on a side (I. Fox, 1940; Hubbard, 1947; etc.) or in stating that the first genal spine is about equal in length to the second (Ewing and Fox, 1943; Holland, 1949, etc.).

Manubrium. Various texts state that the manubrium in *C. felis* is not expanded apically or slightly expanded while in *C. canis* it is broadly expanded. Of the 40 male specimens of *C. felis* from Puerto Rico only four had the manubrium not at all expanded, 21 had the manubrium slightly expanded and 15 had the manubrium moderately expanded as shown in Fig. 2, but in no case was the expansion as great as in the Iowa male specimen of *C. canis* also shown in Fig. 2. These counts are based on the examination of the manubrium of one side, for in general the two sides were alike.

Abdominal spiracles. The diameters of the first, fourth and seventh abdominal spiracles of one side of the Iowa male specimen of *C. canis* measured in microns 48, 28 and 28 respectively. The same spiracles of the 40 male specimens of *C. felis* from Puerto Rico were measured in the same way with the following results: spiracle I varied from 31 to 42 but 65% were under 36; spiracle IV varied from 14 to 20 but 57% were under 17; spiracle VII varied from 14 to 25 but only one measured 14, only two measured 25 and the rest ranged from 17 to 22. The Iowa female specimen of *C. canis* measured as follows: spiracle I, 53; spiracle IV, 39; spiracle VII, 42. The same spiracles of the 50 Puerto Rican female specimens measured as follows: spiracle I varied from 34 to 53 but only two were above 50 and 84% were from 39 to 45; spiracle IV varied from 20 to 34 but only one was 34 and 88% were from 22 to 28; spiracle VII varied from 22 to 33 but only 3 measured 33 and 82% were from 28 to 30.

Hind tibial chaetotaxy. The male and female specimens of *C. canis* from Iowa provided three hind tibiae for study (one leg of the male was broken off) and all three were different. The female had one hind tibia as described by Jordan (1943) with dorsal subapical bristles as shown in Fig. 3, A, but its other leg had the subapical bristles as shown in Fig. 3, B; and these bristles in the hind tibia of the male were as shown in Fig. 3, C. Of the 50 females of *C. felis* from Puerto Rico one-half had hind tibiae which differed on one side from the other. These 50 specimens provided 100 legs for study and there were seven different types of dorsal hind tibial chaetotaxy as shown in Fig. 4, A–G. In most cases the dorsal subapical bristle group consisted of one large bristle and one short seta. When the short seta was adjacent to the base of the large bristle or not removed by more than three-fourths its length the group was considered to be in type A; when the short seta was removed by more than three-fourths its length from the large bristle type B was the designation. Most of the legs were in either type A or type B. In types C and D there were one short seta and two large bristles, while in types E, F and G no short seta was present at all. The actual distribution of the 100 legs was as follows: Type A, 49; B, 33; C, 2; D, 1; E, 9; F, 4; and G, 2. The 40 males of *C. felis* from Puerto Rican dogs, of which 15 had legs which differed on one side from the other, provided 80 hind tibiae for study. In addition to most of the types found in the female there were three more shown in Fig. 4, H, I and J. The actual distribution was as follows: Type A, 41; B, 22; C, 3; D, 0; E, 5; F, 3; G, 1; H, 1; I, 2; and J, 2.

These results show a very wide range of intraspecific variability as regards the genal spines, manubrium, abdominal spiracles and hind tibial chaetotaxy. It is likely that a similarly wide range of variability exists in *C. canis*, and under these circumstances the idea of the occurrence of intermediate forms, possibly as a result of hybridization (Ewing and Fox, 1943), may require revision. At any rate, no one can say what is an "intermediate form" until the variations of *C. canis* are worked out. The proper course for those dealing with cat and dog fleas is clear. Every effort should be made to reach an exact determination and when this can not be done, the inconsistent features of particular specimens should be described.

SUMMARY

Evidence is brought forward to show that *Ctenocephalides canis* (Curtis) does not occur in Puerto Rico, the species involved being *C. felis* (Bouché). This evidence is based on surveys of rats and dogs and on a re-examination of the published records. It is likely that the same situation exists in other places from which the dog flea has been reported. The five important characters which separate the two species were studied in 50 females and 40 males of *C. felis* and the least variable was found to be the shape of the head, a character which alone permits identification of the species; but the relative lengths of the first and second genal spines, the distal expansion of the manubrium, the size of the abdominal spiracles and the hind tibial chaetotaxy are highly variable although within defined limits. Whether forms intermediate between the two species actually occur would depend on a similar study of the intraspecific variations of *C. canis*.

ACKNOWLEDGEMENTS

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BOOKS RECEIVED

Note: Books received for editorial consideration will be intermittently listed. This acknowledgment must be regarded as an adequate expression of appreciation for the courtesy of the author or publisher. Selections will be made for review in the interest of our readers.

Atlas of Medical Mycology. By Emma Sadler Moss, Clinical Professor of Pathology and Albert Louis McQuown, Clinical Assistant Professor of Pathology, Louisiana State University School of Medicine. 245 pp. 248 illustrations. Baltimore, Maryland, The Williams & Wilkins Co., 1953. Price $8.00.

Handbook of Tropical Dermatology and Medical Mycology, Vol. I. Ed. R. D. B. Ph. Simons, Senior Lecturer at Dermatological Clinic of the University of Leyden and Dermatologist in charge at the Civilian Hospital, Amsterdam, with 40 international collaborators. 845 pp. 587 illustrations. Amsterdam, Houston, New York, London, Elsevier Publishing Co., 1952. Price $15.00.

BCG Vaccination, Studies by the WHO Tuberculosis Research Office, Copenhagen, Monograph Series No. 12. By Lydia B. Edwards, Chief of Field Studies, Carroll E. Palmer, Director and Knut Magnus, Assistant Statistician. 307 pp. Geneva, World Health Organization, Palais des Nations, 1953. Price 15/–; $3.00; Sw. fr. 12.–

Textbook of Virology. By A. J. Rhodes, Research Associate, Connaught Medical Research Laboratories and Professor of Virus Infections, School of Hygiene, University of Toronto and C. E. van Rooyen, Research Member, Connaught Medical Research Laboratories and Professor of Virus Infections, School of Hygiene, University of Toronto. 2d edition. 561 pp. 76 illustrations. Baltimore, Maryland, The Williams & Wilkins Co., 1953. Price $8.00.

Human Factors in Air Transportation. By Ross A. McFarland, Associate Professor of Industrial Hygiene, Harvard School of Public Health. 830 pp. illustrated. New York, Toronto, London, McGraw-Hill Book Company, Inc., 1953. Price $13.00.

BOOK REVIEWS

Infektionskrankheiten, Vol. 1 of *Handbuch der Inneren Medizin*, 4th Edition; edited by G. v. Bergmann, W. Frey and H. Schwiegk; in 2 parts: part 1, pp. 1536 with 417 illustrations, some in color; part 2, pp. 1225 with 293 illustrations, some in color. Cloth. West Berlin w. 35, Springer-Verlag (Reichpietschufer, 20), 1952. Price DM 374; by subscription, DM 299.

A collection of excellent monographs makes up the volume on infectious diseases of the fourth edition of the Handbuch der Inneren Medizin. The arrangement of each follows the pattern set by German texts—definition, history, causative agent, epidemiology, pathogenesis, and this is followed by a thorough description of the clinical and autopsy findings, diagnosis, treatment and prevention. Each brings together the knowledge of diagnosis and treatment that has accrued during the past twelve years. The international literature has been carefully and critically analyzed by experts, and there are a great many references. For example, the list of references for the chapter on brucellosis covers twelve pages of close printing. The progress in the field of infectious diseases is accommodated by an expansion in the size of this edition. A very useful author index covers 170 pages and the subject index requires 45. The individual monographs will serve as a means of quick orientation and valuable reference. It fills a need both in the clinic and on the desk of the investigator. The set is superbly printed, and the text is well illustrated with charts, tables and electron microscope pictures of viral agents. The experiences with the newer chemotherapeutic drugs are fully detailed and discussed.

The student of diseases of warm climates will find a great deal of information in the first chapter on general epidemiology, and then in the second part in the well-balanced chapters on leptospirosis, toxoplasmosis and brucellosis. Equally informative are the chapters on protozoan infections with arthropod vectors and on helmiothic infections. These volumes represent documentary evidence of the powerful resurgence of the high scientific spirit of thoroughness in western Germany.

K. F. MEYER

Malaria. Basic Principles Briefly Stated, by PAUL F. RUSSELL, M.D., M.P.H., Division of Medicine and Public Health of The Rockefeller Foundation; Consultant to Surgeon General, U. S. Army; formerly Chairman Expert Committee on Malaria of the World Health Organization, 210 pp. 64 illustrations, 3 in color. Published simultaneously by Blackwell Scientific Publications, Oxford, England; Chas. C. Thomas, Springfield, Illinois, U. S. A. and Ryerson Press, Queen St. West, Toronto 2, Canada, 1952. Price $7.75; 35/-.

This 200-page book on malaria represents a masterly condensation of an extraordinarily complicated and important subject. It succeeds, without ever degenerating into mere synopsis, in covering in a clear, interesting and adequate way the parasite, the mosquito and the disease with its intricate epidemiology and the multiple aspects of its prevention and control. The many photographs and figures are excellent in themselves and well selected to illustrate all phases of the argument.

In order to achieve such conciseness the author had to make difficult decisions as to emphasis and omission. This reviewer would have given some account of the complexes of anopheline species which have caused so much confusion and which the geneticists are now elucidating with success. The discussion of immunology seems inadequate in its treatment of persistent spleens, and of chronicity as a relapse phenomenon rather than the result of overlapping infections; there is too little mention of plasmodial strains, which James thought to be more important in immunology than species, or of the meaning of the parasite formula, based on blood examinations, which is bound to mislead inexperienced malariologists.

On the other hand, only an expert of the widest experience and profoundest knowledge of malaria could have put so much practical and sound information into such small space. The omissions are intentional and doubtless painful sacrifices to the spirit of brevity.

L. W. HACKETT

Review on Comparability of Statistics of Causes of Death According to the Fifth And Sixth Revisions of the International List, Bulletin of the W. H. O., Supplement 4, Geneva, World Health Organization, Palais des Nations, 1952. 2/9; $0.50; Fr. fr. 160.–; Sw. fr. 2.–

The sixth revision of the International List of Causes of Death was accompanied by a drastic change in the method of medical certification of cause of death. The selection of a principle cause is no longer accomplished by means of an arbitrary set of rules but is the responsibility of the certifying physician. As a result of these changes, the continuity of trend series of causes of death has been greatly disturbed and comparison with previous decades extremely difficult. For example, the death rate from diabetes has dropped by more than 50% merely as a result of these changes.

The present booklet was prepared by the W.H.O. Center for Classification of Diseases to indicate the difficulties encountered in maintaining continuity of statistics of causes of death and to provide data which may be useful in achieving comparability. Most useful is a table presenting the International List categories, sixth revision in terms of categories in fifth revision. The detailed comparison of the two revisions is presented for data from Canada. It would be very desirable to have similar tables for many areas of the United States.

J. YERUSHALMY

Ground Equipment and Insecticides for Mosquito Control, edited by E. F. KNIPLING—C. T. Williamson, Business Manager, Yapank, New York, Bulletin No. 2, American Mosquito Control Association, Inc., 1952. 116 pp. Price $2.00.

The American Mosquito Control Association has come forth with another important aid to medical men, engineers and public health workers in general. This AMCA Bulletin No. 2 and its companion Bulletin No. 1, The Use of Aircraft in the Control of Mosquitoes, will help all those engaged in public health work to answer many perplexing problems connected with the selection and use of equipment and insecticides. These contributions are not limited in usefulness to mosquito control but are applicable to many other problems in the field of public health. The discussion of the characteristics and principles of dispersion of insecticides and the classification of sprays and dusts by particle size clears up many of the misconceptions, and should tend to check the present loose usages of terms.

Equipment of various types for dispersing insecticides, including hand atomizers and dusters, blower equipment, aerosol devices and hydraulic pumps are described and illustrated. How to select equipment, insecticide formulations and dosages for various situations is presented in tabular form. Methods of determining relative abundance of different species of mosquitoes are described. Information on the following insecticides and how they are formulated is presented: petroleum oils, pyrethrum, allethrin, DDT, TDE, methoxychlor, BHC, lindane, chlordane, toxaphene, dieldrin and heptachlor. The much discussed question of how to use insecticides safely is effectively covered.

An appendix presents a useful list of manufacturers of equipment, accessories, insecticides and solvents, together with specifications of each of the insecticides and their formulations also a convenient set of conversion tables of weights and measures. A well chosen bibliography of 159 titles is included. As a reference work for purchasing agents, project directors and workers in the field this pair of bulletins is invaluable.

F. C. BISHOPP

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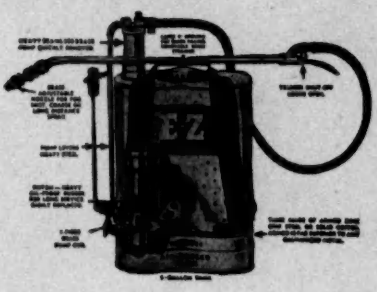

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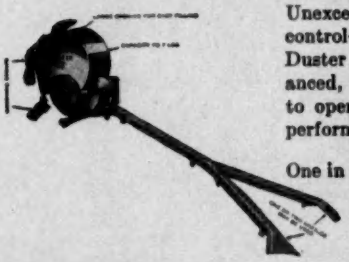

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